PROCEDURES IN STUDYING AND FACTORS INFLUENCING THE QUALITY AND FLAVOR OF ROASTED PEANUTS

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INFLUENCING THE QUALITY AND

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CHAPTER I

INTRODUCTION

Interest in peanut research at Oklahoma State University seems only natural because of the position Oklahoma occupies in the peanut industry. In 1962, Oklahoma was fifth in acreage and sixth in production in the United States. A total of 118,000 acres of peanuts were harvested with an average yield of 1400 pounds of farmer's stock peanuts per acre. This was the highest acre yield in the history of the State for a total of 165,200,000 pounds of peanuts.

This dissertation reports research performed to accomplish three main purposes: (A) to develop new procedures for the determination of the fatty acid and glycerol content of fats and oils by gas chromatography, (B) to isolate and identify precursors of roasted peanut flavor as well as locate their origin within peanut seeds and (C) to isolate and identify the components responsible for typical roasted peanut flavor.

A. New procedures were developed for the analysis of fats and oils for various reasons. Most of the available methods entailed excessive manipulations making the time involved per analysis extremely high. Decreasing this time was necessary to permit analysis of the several hundred samples of oil that were being collected for analysis each year. Also, many of the methods available were designed for qualitative rather than quantitative work. Others, although designed to give quantitative results, were subject to inaccuracies because of losses of some of the

methyl esters during the involved manipulations.

A simple, convenient and quantitative procedure was developed (Chapter III) for the determination of the relative fatty acid contents of fats and oils by gas chromatography. This procedure eliminated the manipulations which resulted in loss of methyl esters, such as aqueous extractions, evaporation of organic solvents used in the extraction of methyl esters from the reaction mixtures and the use of refluxing temperatures. Thus, this procedure differed from available procedures mainly in the way the samples were prepared. Materials used for gas chromatography were essentially those developed by other workers.

An unwanted side-reaction, a study of which is presented in appendix A, caused the formation of a polymeric substance which, if not controlled, interfered with resolution of methyl esters appearing in the early part of the chromatograms and made interpretation of the gas chromatograms difficult or even impossible. Although the study did not reveal the exact mechanism by which polymerization occurred, it did show that the polymer content of the reaction mixtures could be kept sufficiently low by controlling the concentrations of reactants and the catalyst, and the temperature used.

Observations made during the development of this procedure led to a second procedure (Chapter IV) which afforded more information and was free of interfering side reactions, though it was not as convenient or as adaptable to routine analyses of large numbers of samples. This second procedure allowed the concomitant quantitation of both the glycerol and fatty acid content of a fat or oil by gas chromatographic analysis. The quantitation of both moieties of a fat or oil is not provided for by previously available methods.

B. The word "flavor" has had a variety of meanings as it has been used by workers in the fields of food acceptance and flavor chemistry. For the purpose of this dissertation, the word "flavor" will cover the sensory responses of the taste buds as well as the olefactory nerve. Responses from other sensory organs of the mouth and the visual responses are purposely excluded from this definition because precursor isolation necessarily involves the destruction of the original texture and visual character of peanuts. Consequently, these responses are not particularly applicable to studies reported here.

A knowledge of the locale within the seed and chemical nature of the compounds which give rise to peanut flavor during roasting was considered essential to any research program designed to improve peanuts genetically or to improve the quality of roasted peanuts or peanut products.

Research of this type involves some characteristic problems. The means by which the isolations were to be accomplished had to be carefully chosen and assessed because of the possibility that the flavor precursor was a multicomponent system. If this were the case, no single precursor would give rise to typical flavor; rather, several combinations of component parts would have to be made in the hope of obtaining just the right combination for flavor development during roasting. The meager evidence available in the literature suggested that peanut flavor resulted from a combination of common molecular species known to be present in peanut seeds such as the reserve proteins, amino acids and saccharides. By inference from what is known of the morphology of the quiescent state of peanut seeds and from circumstantial evidence gained from roasting studies (Chapter V), it was concluded that the flavor precursors were,

contrary to common belief, most likely specific molecular species capable of being separated as an individual fraction. With this information, two different approaches were taken (Chapter V): one designed to isolate the precursors and the other designed to localize them.

C. Knowledge of the compounds responsible for typical peanut aroma and taste was deemed essential to any research program designed to improve and stabalize the flavor of roasted peanuts and peanut products, such as peanut butter.

Well accepted techniques and procedures with some modifications were used for this purpose. Modifications of two common extraction techniques, degassing and steam distillation, were used to extract the flavor components. Fractional distillation at low pressures and temperatures was used to obtain flavor concentrates or composites and to give partial separations of the components of these concentrates. Preparative gas chromatography was used in attempts to obtain complete fractionation of individual components. Nuclear magnetic resonance (NMR), infra-red, and mass spectrometry were used to aid in the identification of the flavor components. The use of "wet-chemical" procedures was out of the question because of the extremely small amounts of material involved in these flavor composites or concentrates. A discussion of these procedures and the results obtained with their use is presented in Chapter VI.

CHAPTER II

LITERATURE REVIEW

A. Procedures for the gas chromatographic analysis of fatty acid contents of fats and oils.

The first use of gas chromatography in the separation of fatty acids was reported by James and Martin (1) in 1952. They described the separation of carboxylic acids from formic through dodecanoic on silicone-stearic acid columns and detected the eluted acids by titration. In 1953, Cropper and Heywood (2) extended the gas chromatographic separation to include methyl esters of even-numbered carbon fatty acids from C_{12} to C_{22}. These authors used a platinum wire thermal detector. James (3) and Stoeffel et al. (4) used Apiezon paraffins for the liquid phase in separation of high molecular weight fatty acid esters. This was not the first reported use of Apiezon, but represented considerable improvement in separation due to improved techniques of coating the solid support with the liquid phase. The Apiezon paraffin waxes and the silicone greases provided good separation of the fatty acid methyl esters of different chain length but did not provide satisfactory separation of olefinic fatty esters. Also, despite the presence of optimal conditions, the elution of fatty acid methyl esters of chain lengths up to 20 carbons took as much as three to four hours. The bands emerging near the termination of the chromatogram were poorly resolved and showed typical diffusion or broadening of the peaks.

Lipsky et al. (5, 6) and Orr and Callen (7) reported on the use of copolymers of dibasic acids with di- or polyglycols having molecular weights in the range of 3 to 6 thousand as liquid phases for the separation of fatty acid methyl esters. Using various copolymers, good separations of olefinic fatty esters of the same chain length were obtained. Since the publication of these two papers, improved copolymers have been produced which are less susceptible to the "ghosting" and transesterification referred to by Orr and Callen (7) and Lipsky and Landowne (6), and can be used with good results at lower temperatures. Thus, the newer polymers are less susceptible to "bleeding" of the liquid phase from the column which often accompanies the use of higher temperatures.

Thus, the basic procedures and materials to be used for the successful gas chromatographic separation of methyl esters of fatty acids were well established by 1960. More recent improvements such as better liquid phases, the introduction of Golay (8) columns and the advent of programmed temperature chromatographs have all improved this particular phase of gas chromatography.

Procedures for preparing the methyl esters from free fatty acids and directly from lipids for gas chromatography have become numerous since James' first report in 1952. A number of authors have described methods for the methylation of fatty acids after their separation or release from lipid materials by saponification or hydrolysis. James (9) used acid catalysis and diazomethane for the formation of esters from fatty acids in excess methanol, Roper and Ma (10) used diazomethane, Hornstein et al. (11) employed conversion of acids fixed to Amberlite IRA-400 ion-exchange resin, Lorette and Brown (12), Hajra et al. (13, 14), and Ackman et al. (15) used 2,2-dimethoxypropane in excess methanol under

acidic conditions to remove the water of reaction during esterification, Metcalf and Schmitz (16) used BF_3 as the catalyst in excess methanol, and more recently Gehrke and Goerlitz (17) used silver oxide and methyl iodide to form methyl esters after saponification. Stoeffel et al. (18) used a microsublimation technique to remove fatty acid methyl esters from the nonsaponifiable contaminants.

Some of these procedures are not strictly comparable because they were formulated for specific purposes. However, Vorbeck et al. (19) have recently compared three of the most applicable of these procedures; namely, those of Stoeffel et al. (18), Metcalf and Schmitz (16) and Roper and Ma (10). Only the diazomethane procedure was found to give nearly quantitative recoveries of all the methyl esters. Metcalf stated that his procedure was not intended to be absolutely quantitative. Instead, it was designed to give rapid results for quality control work. Stoeffel et al. specified that their microsublimation procedure was to be used for the higher molecular weight esters. Work in this laboratory confirmed that it could not be used with samples containing short chain fatty acid esters because of losses under vacuum. Apparently, diazomethane is the preferred reagent for quantitative work although the procedure of Gehrke and Goerlitz gives excellent results also. Yet, both of these procedures also have disadvantages. Diazomethane is toxic, explosive, must be freshly prepared at each use and may result in the formation of pyrazolines at double bonds (20). The use of silver oxide and methyl iodide apparently avoids all these difficulties, but does involve a large number of manipulations. From this standpoint, BF_3 and 2,2-dimethoxypropane procedures are probably the simplest and easiest to use.

Procedures describing direct preparation of methyl esters from lipid

materials without prior saponification or hydrolysis which take advantage of trans- or interesterification have also been published. Craig and Murty (21) used sodium methoxide and refluxing times of about 30 minutes. Luddy et al. (22) improved on this procedure by using potassium methoxide. Reflux times were reduced to about 5 minutes even for transesterification of the more difficult steryl esters. Once again, the numerous manipulations involved in the use of this procedure and the loss of lower molecular weight esters during refluxing made adaption of this procedure to routine analysis of large numbers of samples extremely difficult without employing considerable technical assistance.

Tove (23) and Waller (24), interpolating from the use of 2,2-dimethoxypropane in preparation of methyl esters from free fatty acids, reported the use of 2,2-dimethoxypropane for the transesterification of lipids in methanol under acidic conditions at room temperatures but did not report data to establish quantitative recoveries using this reagent. Peterson et al. (25) have recently reported the use of BF_3 and BCl_3 for transesterification of glycerides.

B. Isolation and identification of the components and precursors of roasted peanut flavor.

Literature pertaining to this subject is notably lacking. It is apparently completely limited to the work of Pickett and Holley (26) and a review by Hoffpauir (27) which presented views based mostly on speculation. Among the findings of Pickett and Holley was the fact that carbon dioxide made up 98% of the gases evolved during roasting. Also, positive tests for aldehydes, ammonia, sulfur compounds and diacetyl were obtained.

These authors also studied the changes which took place in the protein, carbohydrate and lipid fractions of peanuts during roasting.

Lipids, it had been found earlier (26), underwent little or no change during roasting. Free amino nitrogen content changed significantly while total nitrogen content was not measurably altered. Reducing sugar and starch content remained fairly constant during roasting but sucrose content dropped from about 9.5% to 0.5% of the weight of the peanuts. Experiments were performed which showed that sucrose and amino acids would react at temperatures at which peanuts were roasted (140° C.) to evolve carbon dioxide and form brown residues. Whether this is what actually occurs in peanuts during roasting is purely conjecture. Hoffpauir summarized what was known in 1953 about the precursors of roasted peanut flavor as follows:

> The sugar probably undergoes caramelization to some extent and at the same time reacts with free amino groups of both protein and free amino acids to produce nonenzymatic browning. The browning reaction is extremely complex and its mechanism is not thoroughly understood. It is known, however, that the reaction of sugar with amino acids produces characteristic flavors and aromas. The products evolved in this reaction include carbon dioxide and furfural derivatives both of which have been identified among products volatilized when peanuts are roasted. When extensive browning occurs there is usually a loss of nutritive value of the protein.
>
> The polysaccharides present, such as the arabans, are degraded and possibly decomposed by heat. There is also probably some thermal decomposition of the protein since sulfur compounds have been identified in the volatile matter. The characteristics of the oil undergo little, if any, change but the lower viscosity of the heated oil allows it to penetrate and wet all parts of the kernel.

Since the time of this review, nothing in the literature has added significantly to this sketchy picture of what happens during roasting to produce flavor.

Procedures for isolating and identifying volatile flavor components are numerous and varied. These often involve a combination of trapping out the volatile substances at low temperatures and making separations and identifications by gas chromatography and mass spectrometry. Since

the development of gas chromatography, the volatiles from a large number of foods have been studied. Weurman (28) has separated the components responsible for raspberry aroma and Bailey et al. (29) studied the enzymatic release of isothiocyanates from cabbage using gas chromatography and mass spectrometry. Issenberg and Wick (30) obtained an odor concen- from bananas on a falling-film evaporator, analyzed the vapor from the concentrate in the head space of sealed containers using gas chromatography, made preparative gas chromatographic separations from the concentrate and identified many of the individual components with infra-red spectroscopy. Bailey et al. (31) used gas chromatography for separation of the volatiles of cocao beans, Bassette et al. (32, 33) and Patton (34) for off flavors in milk, Bernhard (35) for volatiles of essential oils of oranges, Jackson and Hussong (36) for alcohols and methyl ketones in Blue cheese, Jennings and Creveling (37) for the study of the volatile esters of Bartlet pears, Kramlich and Pearson (38) for volatiles of cooked beef flavor, Merritt et al. (39) and Wick et al. (40) for volatiles of irradiated beef, Paulicek et al. (41) for the study of odorous substances of honey, Rhoades (42), and Zlatkis and Sivetz (43) for the volatile constituents of coffee and Vorbeck et al. (44) for sauerkraut volatiles. Other literature may be cited but these are references representative of the various applications of gas chromatography to flavor and aroma problems.

Obviously, before gas chromatography may be performed, some means for removing the volatile constituents from the substance being studied must be available. There are three common means of accomplishing this removal which have been modified for many purposes. Steam distillation, vacuum degassing or film evaporation and head-space analysis are the

three most used and examples of their use may be found in the literature dealing with the use of gas chromatography in flavor problems.

Excellent techniques for the vacuum isolation, bulb to bulb distillation and mass spectrometric analysis of volatile constituents have been reported by Bazinet and Merritt (45), and by Merritt et al. (39).

There are no published accounts of attempts to isolate flavor precursors from raw peanuts. In fact, the only thorough attempts to isolate precursors of flavor of any kind have been made on meat, fish and some vegatables. Beef has been studied by Batzer et al. (46) and Hornstein et al. (47) while fish flavor precursors have been studied by Fujita and Hashimoto (48). Considerable information has been learned of the precursors of typical flavor of the Cruciferae family (cabbage, mustard, turnip, etc.) and allium genus (onions, garlic, etc.) (49). Work that aided considerably in formulating procedures for the isolation and localization of peanut flavor precursors has been published. The procedures of Naismith and McDavid (50) were used to make extracts containing the flavor precursors. The non-aqueous, density gradient procedures for particulate fractionation used by Diekert et al. (51, 52) have been used with success for the localization of peanut flavor precursors within peanut cotyledon parenchyma cells. Woodroof and Leahy (53) have published information on parenchyma cell morphology which contributed to the formulation of particulate fractionation procedures.

CHAPTER III

DIMETHOXYPROPANE INDUCED TRANSESTERIFICATION OF FATS AND OILS

Several methods have been devised for preparing fatty acid methyl esters from glycerides. Craig and Murty (21) described a method employing sodium methoxide as the catalyst and reflux times of the order of 30 minutes. Luddy et al. (22) showed that the reflux time could be shortened to 5 minutes by using potassium methoxide as the catalyst. Under these conditions even the steryl esters could be completely transesterified. Recently, Peterson et al. (25) described a method which employed boron trichloride as the catalyst and a prescribed heating period. Gehrke and Goerlitz (17) prepared the esters by treatment of the saponified lipids with silver oxide and methyl iodide.

The use of 2,2-dimethoxypropane (DMP) in preparing methyl esters from lipid materials was reported by Tove (23) in studies on epidermal fats of rats and by Waller (24) in studies on peanut oils, but data to support the quantitative conversion of glyceride fatty acids to methyl esters were not presented by either author.

Work in this laboratory indicated loss of the esters could occur when reaction mixtures were refluxed, when the esters were extracted over aqueous phases, and when solvents were removed by evaporation. This loss was especially pronounced with the lower molecular weight esters.

Consequently, attempts were made to devise a procedure free of

time consuming and error ridden manipulations involving refluxing, evaporations or extractions over aqueous phases. The described procedure is especially adapted to the routine analyses of large numbers of samples by gas-liquid chromatography (GLC).

APPARATUS AND REAGENTS

Apparatus. GLC analyses were performed with an F and M model 500 linear programmed gas chromatograph equipped with a Disc Instruments, Inc., disc integrator, Bristol 1 mvolt recorder, and a hot wire detector. Most of the chromatograms were obtained with 1/4 inch by 8 foot aluminum columns of 15% Lac-3R-728 on Chromosorb W, 60 to 80 mesh. However, some chromatograms were obtained with 1/4 inch by 6 foot aluminum columns of 14.5% EGS on Anakrom, 100 to 110 mesh, type A. Helium at a flow rate of 60 ml. per minute was used as the carrier gas.

Reagents. Lac-3R-728 (Cambridge Industries Company, Inc.).

Anakrom, 100 to 110 mesh, type A, (Analabs, Inc.).

EGS, ethylene glycol succinate (Applied Science Laboratories).

Benzene, reagent grade, dried over sodium.

2,2-Dimethoxypropane (Dow Chemical Company) redistilled from 76 to 79° C.

Methanol, super-dry with less than 0.02% water (British Drug Houses, Ltd.).

Triglycerides, 99.5% purity (Mann Laboratories) used without further purification.

Methanolic HCl: Bubble dry HCl gas through cooled methanol until the concentration is in excess of 10% (w./v.) as noted by titration with standard base. The solution is stored in the cold over a desiccant and re-titrated periodically to check its strength.

Neutralizing Agents: (1) sodium bicarbonate, sodium carbonate and sodium sulfate (anhydrous) 2:1:2 ratio by weight, dried overnight at 110° C. and stored in an air-tight container. (2) Approximately 0.5N sodium methoxide in methanol, made by reacting sodium with super-dry methanol.

Standard methyl esters (Applied Science Laboratories), chromatographically pure, used without further purification.

PROCEDURES

Preparation of Esters From Pure Triglycerides. To obtain time of reaction data and recovery values with standard triglycerides, the procedure was as follows: individual triglycerides were weighed separately in porcelain boats and combined in 50-ml. glass stoppered Erlenmeyer flasks. Reagents were added in the following order: 14 ml. of dry benzene, 1 ml. of DMP and 5.0 ml. of methanolic HCl of the desired concentration. Two-ml. aliquots were removed periodically and delivered to glass stoppered 10-ml. Erlenmeyer flasks containing approximately 0.25 gram of the solid neutralizing mixture. Finally, the samples were swirled to insure complete neutralization and allowed to stand 30 minutes before 50-µl. aliquots were withdrawn for GLC analysis.

Preparation of Esters From Fats and Oils. Approximately 0.200 gram samples were accurately weighed in porcelain boats and placed in 50-ml. glass stoppered Erlenmeyer flasks. Volumes of reagents were the same as those used for triglycerides and they were added in the same order. Ten percent methanolic HCl was used. The reaction mixture was allowed to stand overnight at 22° C. to insure complete transesterification. The entire sample was neutralized with approximately 2 grams of the solid neutralizing mixture and after 30 minutes 50-µl. aliquots

were withdrawn for GLC analysis.

RESULTS AND DISCUSSION

Isopropylidene Glycerol (IPG) Formation During Transesterification. Lorette and Howard (54) described the preparation of DMP from IPG under conditions which suggested to us that in the presence of acid, excess DMP, and methanol, glycerol resulting from transesterification of lipids would be converted largely to IPG. IPG was synthesized according to the procedure of Newman and Reynold (55) and purified using preparative GLC. This material was used to determine the retention time of IPG in gas chromatograms of the fats and oils and the triglyceride mixtures.

The presence of IPG in the transesterification mixtures was confirmed as follows: two-ml. aliquots were removed periodically from a triacetin reaction mixture, neutralized and the solvents removed at a pressure of 2 mm. Hg. Infra-red spectral analysis of the small amounts of residue remaining showed the development of the IPG spectrum with time up to about 6 to 8 hours. At this time the spectrum of the residue was essentially that of IPG. Large volumes of the transesterification mixtures were prepared, allowed to stand for 24 hours at 22° C., neutralized and filtered. The solvents were removed under vacuum and the residues fractionally distilled at 2.5 mm. of mercury. Mass spectral analyses of the fractions obtained confirmed that IPG was the major end-product with very small amounts of 4,4-dimethylcyclo-3,4-dioxahexan-1-ol also present.

Polymer Formation During Transesterification. Formation of a yellow substance during the time alloted for transesterification resulted in a series of unwanted peaks appearing in chromatograms of the esters.

A study of the parameters affecting the rate of formation of this colored substance revealed that an increase of temperature, HCl concentration, or DMP concentration measurably increased its formation. Since relatively high concentrations of HCl were absolutely necessary for complete transesterification, the only alternative was to reduce to a minimum the temperature and the DMP concentration used. Consequently, the amounts of reagents used and temperature employed in the final procedure were in part a result of these studies.

Elemental analyses and infra-red spectra of this yellow substance indicated that it was a polymer originating from DMP and resulting from condensations involving the loss of the elements of water.

Necessity of DMP for Complete Transesterification. Figure 1 contains the results of studies showing that in the absence of DMP, transesterification of triglyceride mixtures at 22° C. was not complete over the time intervals studied. Further, when the mixtures lacking DMP were analyzed as much as 24 hours later transesterification was still incomplete. This was confirmed by adding DMP to these samples after extended periods of time and re-analyzing them. When this was done, transesterification was increased measurably in every case and was always nearly 100% complete.

These data were also indicative of the effect of the formation of IPG on transesterification. Notably, the final spurt of transesterification corresponded closely to the maxima in the curves representing IPG formation suggesting that a considerable amount of glycerol had to be converted to IPG before the driving effect on transesterification was observed. This interpretation was also supported by extrapolation of the curves representing transesterification in the presence of DMP

from the point at which the final spurt began. Curves resulted which coincided with those representing transesterification in the absence of DMP. The extrapolations are represented by the dotted lines in Figure 1. These effects, which were qualitatively the same in both levels of methanolic HCl used, differed primarily in magnitude.

Effect of Temperature on Transesterification. Data plotted in Figure 2 show that at temperatures below 22° C. the time necessary for complete transesterification increased rapidly. The reaction was not complete at 5° C. even after several days as indicated by the dotted line extension of the curve. Temperatures above 22° C. decreased the time necessary for completion and at 90° C. the reaction was complete within 45 minutes. However, temperatures above 22° C. were avoided because of volatility of reagents and esters and increased polymer formation in the reaction mixtures. Also, at 22° C. rigid control of temperature is not necessary, and by proper choice of HCl concentration the reaction time (6 to 8 hours) may be overnight thus allowing the evening preparation of large numbers of samples which are ready for analysis the following morning.

It must be emphasized that higher temperatures could be used if time were of prime importance; however, resolution in the shorter chain ester (C_4 to C_{12}) region of the gas chromatogram was adversely affected due to greater polymer content of the reaction mixture. Resolution in the longer chain ester (C_{14} and higher) region was not affected as long as the reagents were used as specified in this procedure.

Effect of Methanolic HCl Concentration on Transesterification. The effect of the methanolic HCl concentration on complete transesterification and IPG formation is shown by the recovery values in Table I.

Recoveries of fatty acids determined by GLC analysis were satisfactory at methanolic HCl concentrations of 5.4% to 12%. Mixtures were used which contained most or all of the following triglycerides: tricaproin, tricaprylin, trilaurin, tripalmitin and tristearin. Although IPG formation was noticably variable and incomplete, it served as a convenient marker for determining retention times in subsequent chromatograms.

It was possible to establish the optimum concentration of HCl in methanol to be used by studying the time necessary for complete transesterification as a function of concentration of HCl in methanol (Figure 2). Concentrations up to about 10% greatly decreased the time necessary for complete reaction whereas higher concentrations were relatively less effective. A concentration of 10% HCl in methanol was chosen for this procedure because: (1) solutions of higher concentration weakened rapidly even when refrigerated. (2) Complete transesterification within overnight reaction time (6 to 8 hours) was obtained. Lower concentrations of HCl may be used but an appropriately longer time for transesterification must be allowed. This time may be found from the curve in Figure 2.

Discussion of Neutralization Procedures. When the solid neutralizing mixture was used, a negative volume change occurred, the magnitude of which was difficult to determine precisely in routine analyses. Consequently, recovery values were more variable and less accurate. As long as only relative values of fatty acid composition were desired, this neutralization procedure was preferred since it was simpler and more rapid. However, if absolute amounts of fatty acids were desired, sodium methoxide neutralization was preferred since no appreciable volume change was observed using this reagent and the final dilution of the

sample was obtained by addition of the volumes of reagents added. Consequently, less variable recovery values were obtained using this reagent. Equally satisfactory recovery values using the solid neutralizing mixture were not obtained unless the supernatant was decanted into a 25-ml. volumetric flask, and the volume adjusted to 25 ml. before GLC analyses were made.

Accuracy and Precision. Table II contains recovery values obtained from three different triglyceride mixtures using sodium methoxide neutralization. Approximately 30 μmoles each of tricaprylin, trimyristin, tripalmitin, tristearin and triolein were used to supply about 450 μmoles total esters after transesterification. This corresponded closely to the total μmoles of ester obtained from 0.150 to 0.200 grams of a fat or oil. Standard deviations ranged from 2.69 for caprylate to 0.48 for myristate with average recoveries being very nearly 100% of that added as the triglyceride. These data clearly indicated the accuracy of the procedure and the efficacy of performing triplicate determinations to minimize error.

Fatty acid contents of various fats and oils obtained using this procedure are tabulated in Table III along with standard deviations from the mean based on duplicate determinations. Standard deviations ranged from 0.00 to 3.62 for a relative error under ± 5% in most cases. However, the standard deviations are of the same order for lesser components thus increasing considerably the relative error for these. Precision in measuring lesser components should be improved using the more sensitive ionization detectors because most of the error was introduced by the disc integrator in measuring peaks below ten units deflection on the chart. The error was especially high if the peaks were both low and

spread out. Thus, errors of ±20% were observed with lesser components emerging in the latter part of the chromatograms.

Nearly 200 samples of peanut oils were analyzed using this procedure and mean fatty acid contents from two replications of each of ten of these oils are tabulated in Table IV along with over-all means and fiducial limits at the 5% level of probability. With the exception of one value (C_{22} for sample number 2), analysis of variance showed no significant difference from the mean within each group of fatty acids at the 5% level of probability. These data showed that where large numbers of samples of the same type of oil were analyzed, close confidence limits were established with a high level of probability. Values for lignoceric acid (C_{24}) were not determined because under the conditions of GLC analysis used, lignocerate had a retention time of about 80 minutes and was so broad and low that its quantitation was impossible.

Calculations. Amounts of fatty acids determined were calculated from the gas chromatograms by comparing the integrated areas obtained from transesterified triglycerides or fats and oils to those obtained from a mixture of appropriate methyl esters in known concentrations. Increments of the known solution of methyl esters were injected and areas plotted versus μmoles injected to assure linearity of response of the detector for the amount of each sample being analyzed.

However, when analyses of the peanut oils were performed, only relative amounts of fatty acids within an oil were of interest. Therefore, relative area-to-μmole relationships of the standard methyl esters were used to calculate methyl ester contents of the oils without using internal standards. In this way, once the relative relationships were determined it was not necessary to re-run standard curves. Nevertheless,

the standard solutions were chromatographed each day that analyses were performed over a period of approximately one year. The absolute area-to-μmole relationships varied considerably with changes in flow rate, resistance of the detector filaments, temperature and program rate, but relative relationships were surprisingly consistent.

Column Conditions. Contained in Figure 3 is a chromatogram of coconut oil on a Lac column. This represented the most difficultly resolvable group of methyl esters of all the fats and oils analyzed. We were not able to analyze coconut oil esters on EGS columns because of the incomplete separation of C_{12} and IPG using this liquid phase.

Values in Tables I and III were obtained with Lac columns using the temperature program outlined in Figure 3, whereas values in Table II were obtained with EGS columns by injecting at 85° C. and programming immediately to 200° C. at 11° per minute. Peanut oil fatty acid contents (Table IV) were obtained with EGS columns operating isothermally at 187° C. More reproducible results were obtained using EGS columns because of the lack of prominent "ghosting" (7) that occurred when Lac columns were used.

SUMMARY

A simple, convenient and quantitative procedure for the preparation of methyl esters from fats and oils, especially applicable where large numbers of samples must be analyzed, has been described. Reaction conditions are flexible; however, the suggested optimum conditions are best used to avoid undesirable side reactions. Linear programming techniques have been used successfully to separate isopropylidene glycerol from C_{12} methyl esters and to resolve lower molecular weight

esters. Although recovery of isopropylidene glycerol is not quantitative, it serves as a convenient marker for determining retention times of methyl esters.

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TABLE I

EFFECT OF METHANOLIC HCl CONCENTRATION ON COMPLETE TRANSESTERIFICATION AND IPG FORMATION

% HCl in Methanol	% Recovery of Fatty Acids and IPG from Mixtures of Triglycerides						
	C_6	C_8	C_{12}	C_{16}	C_{18}	$C_{\overline{18}}$	IPG
2.4	101	94	91	-	99	-	87
5.1	99	102	102	-	96	-	80
8.0	-	95	109	102	100	108	68
10.0	-	99	112	99	101	105	66
12.0	99	96	97	101	100	96	62

TABLE II

ACCURACY STUDY ON FATTY ACID CONTENT OF MIXTURES OF PURE TRIGLYCERIDES

	% Recovery of Fatty Acid Added as Triglyceride				
Analysis	C_8	C_{14}	C_{16}	C_{18}	$C_{\overline{18}}$
1	95.4	100.2	99.8	99.5	100.4
2	99.2	100.4	98.8	99.4	98.1
3	100.6	99.5	98.5	97.2	101.3
Mean	98.5	100.0	99.0	98.7	99.9
Std. Dev.	2.69	0.48	0.75	1.30	1.64

TABLE III

PRECISION STUDY: FATTY ACID CONTENT OF VARIOUS FATS AND OILS

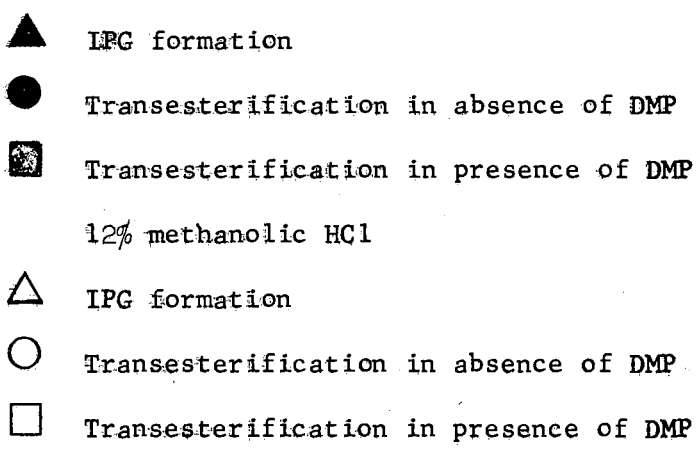
	Mole % and Standard Deviation									
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	$C_{\overline{16}}$	C_{18}	$C_{\overline{18}}$	$C_{\overline{\overline{18}}}$	$C_{\overline{\overline{\overline{18}}}}$
Coconut Oil	12.5 ±1.06	7.3 ±1.13	48.1 ±2.49	16.6 ±3.49	7.6 ±1.84	1.0 ±0.04	3.8 ±0.28	0.9 ±0.14		
Palm Oil				1.1 ±0.54	42.9 ±3.62		3.2 ±0.28	40.1 ±0.31	11.5 ±0.28	
Lard				0.9 ±0.45	27.6 ±0.78	2.6 ±0.0	8.6 ±0.14	41.7 ±0.50	12.3 ±0.34	1.4 ±0.56
Tallow				3.8 ±0.20	29.4 ±2.12	4.8 ±1.03	21.6 ±0.22	39.0 ±2.12	1.3 ±0.22	
Cottonseed Oil				0.9 ±0.0	22.8 ±0.64		2.6 ±0.20	18.5 ±0.32	54.3 ±0.11	
Soybean Oil					11.6 ±0.36		4.4 ±0.85	24.0 ±1.13	56.2 ±1.13	4.5 ±2.12
Hardened Soybean Oil					13.7 ±1.56		5.1 ±0.49	62.2 ±1.77	4.5 ±1.77	
Safflower Oil					8.2 ±0.28		2.3 ±0.22	13.8 ±0.64	75.9 ±0.70	
Corn Oil					12.2 ±1.06		1.5 ±0.0	29.4 ±0.10	56.8 ±0.80	

TABLE IV

MEAN FATTY ACID CONTENT OF SEED OIL FROM TEN DIFFERENT SPANISH PEANUT SELECTIONS

	Mole %						
Sample	C_{16}	C_{18}	$C_{\overline{18}}$	$C_{\overline{\overline{18}}}$	$C_{\overline{\overline{\overline{18}}}}$	C_{20}	C_{22}
1	14.01	3.13	46.16	33.86	0.60	0.72	1.53
2	15.63	3.48	41.69	34.40	0.70	1.10	3.16
3	15.06	2.54	43.54	35.99	0.51	0.67	1.72
4	15.02	2.70	41.68	36.93	0.45	0.84	2.40
5	14.20	2.81	43.52	35.65	0.71	0.68	2.12
6	15.01	2.95	40.93	37.17	0.72	0.90	2.32
7	14.42	2.85	43.64	35.64	0.49	0.52	2.46
8	15.13	3.25	42.35	36.42	0.60	0.74	1.54
9	14.14	3.54	41.52	37.19	0.73	1.00	1.90
10	14.38	3.29	45.60	34.09	0.41	0.87	1.37
Mean	14.70	3.05	43.06	35.76	0.59	0.80	2.05
Fiducial Limit	±2.14	±0.91	±4.19	±3.95	±0.17	±0.17	±0.90

Figure 1. Transesterification in the presence and absence of DMP with two different concentrations of methanolic HCl and IPG formation in the presence of DMP



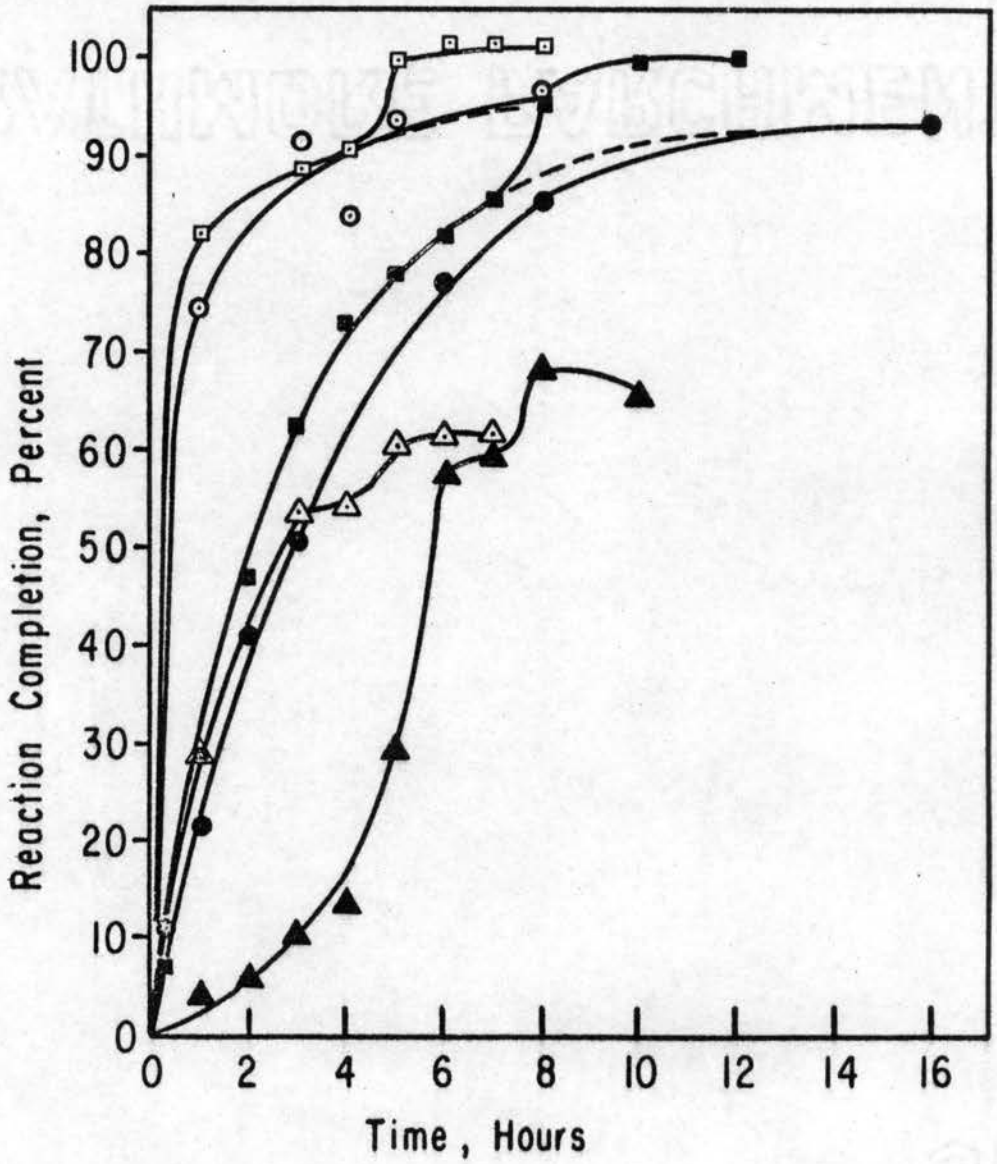

Reaction Completion, Percent
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Time, Hours

Figure 2. Time necessary for complete transesterification as a function of both temperature and methanolic HCl concentration

△ Percent HCl in methanol, at 22° C.

○ Bath temperature, using 10% methanolic HCl

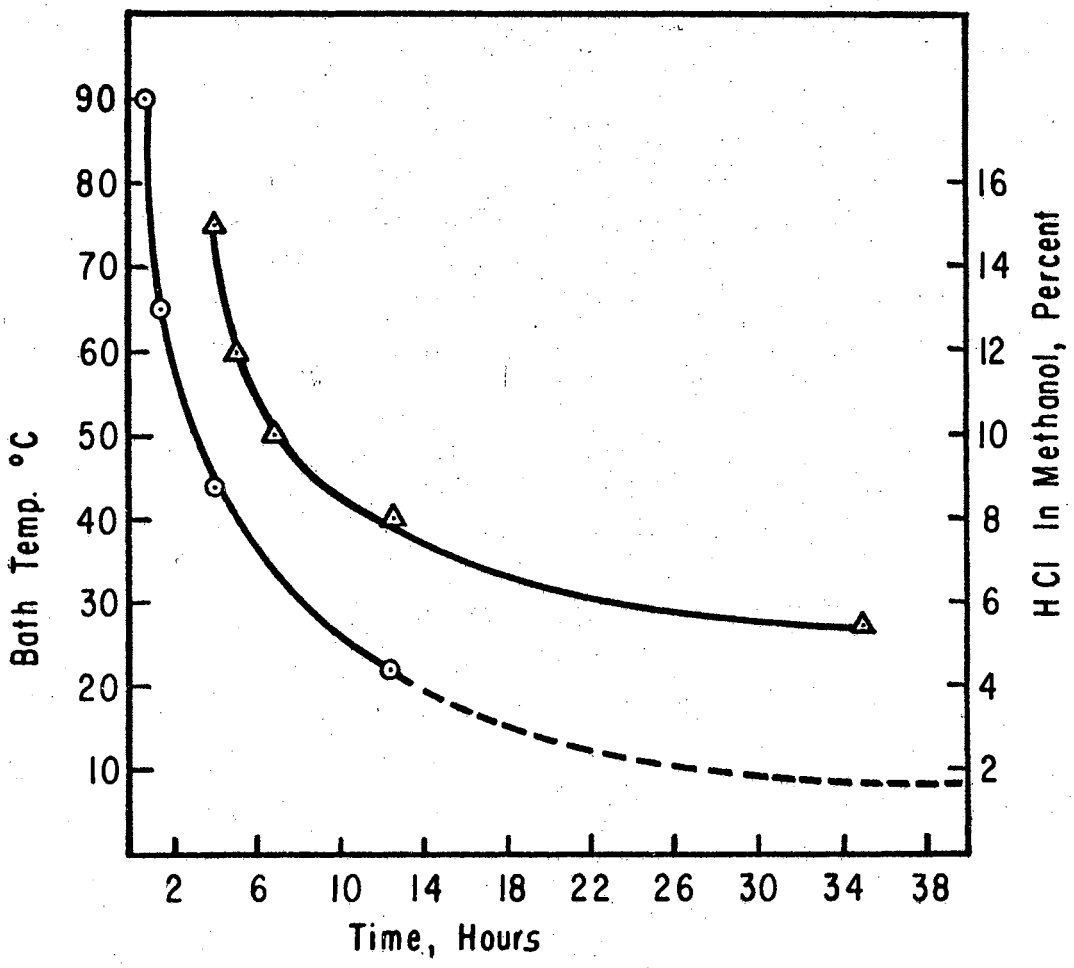
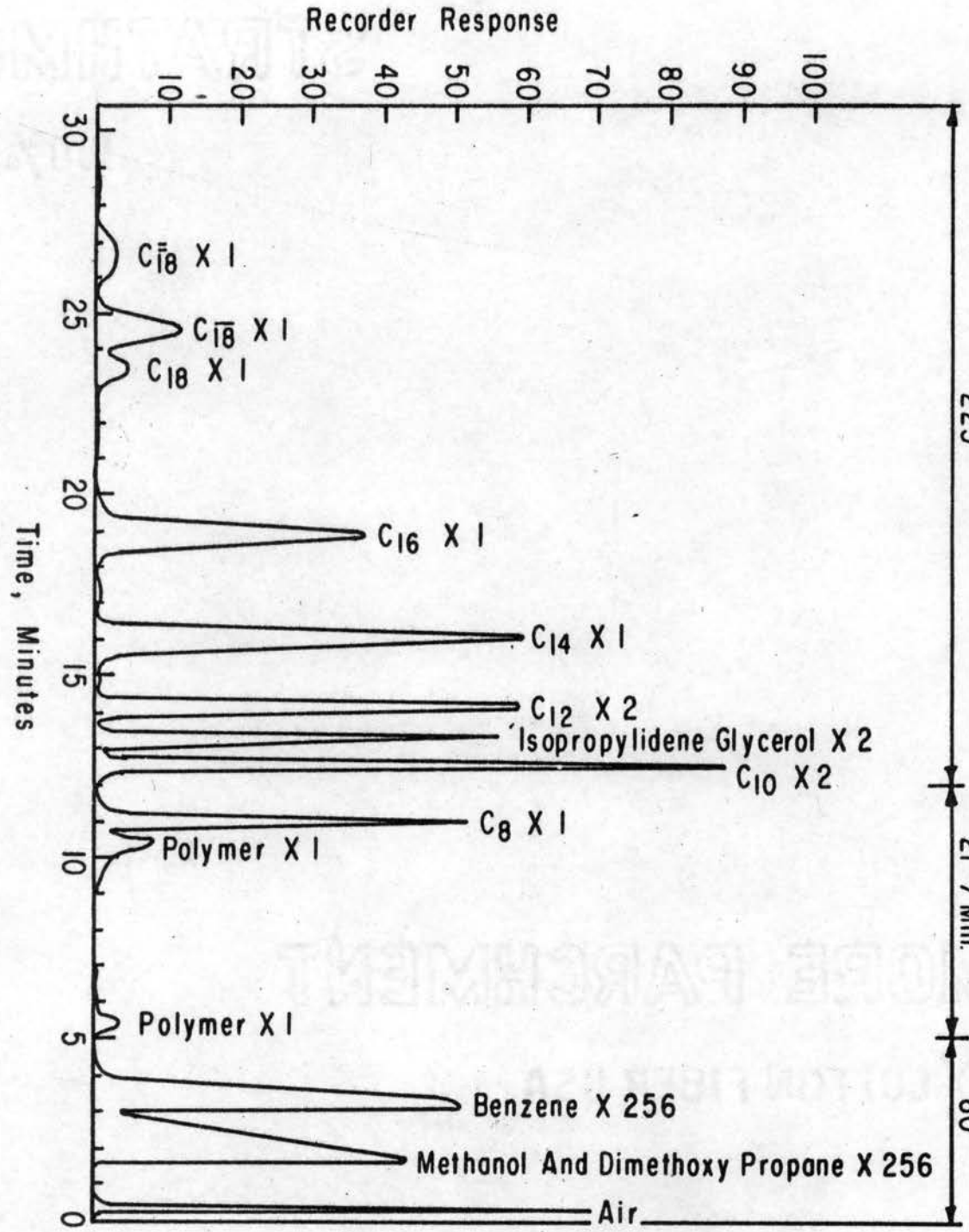

Bath Temp. °C
90
80
70
60
50
40
30
20
10
2
6
10
14
18
22
26
30
34
38
Time, Hours
16
14
12
10
8
6
4
2
HCl In Methanol, Percent

Figure 3. Gas chromatogram of coconut oil on Lac column showing the temperature program used and typical distribution of IPG and the methyl esters


Recorder Response
10
20
30
40
50
60
70
80
90
100
Time, Minutes
30
25
20
15
10
5
0
$C_{\overline{18}}$ X 1
$C_{\overline{18}}$ X 1
C_{18} X 1
C_{16} X 1
C_{14} X 1
C_{12} X 2
Isopropylidene Glycerol X 2
C_{10} X 2
C_8 X 1
Polymer X 1
Polymer X 1
Benzene X 256
Methanol And Dimethoxy Propane X 256
Air
225°
21° / Min.
80°

CHAPTER IV

SIMULTANEOUS DETERMINATION OF GLYCEROL AND FATTY ACIDS

Hornstein et al. (11) described the use of an internal standard to correct for errors introduced into fatty acid determinations by various manipulations before and during gas-liquid chromatography (GLC). Fatty acid values determined by GLC were arbitrarily corrected by a factor determined from the percent recovery of the internal standard. Tinoco et al. (56) have presented evidence indicating that this procedure is valid for some lipid materials. However, internal standards cannot correct for fatty acids which are incompletely released from the lipid or incompletely converted to methyl esters. Errors from incomplete conversion are quite probable with some procedures (19). Obviously, the arbitrary adjustment of values for all methyl esters according to the loss incurred in the internal standard would be valid only in those cases in which the same loss occurred in all the methyl esters present. Seemingly, this occurrence is unlikely in samples containing a diverse representation of both low and high molecular weight esters. Therefore, the degree of error produced by making the arbitrary adjustment would depend on which method is used, the manipulations involved and the type of lipid being analyzed.

The preparation of methyl esters from fats and oils for determination by GLC using a procedure which was extremely simple and required little effort for the preparation of samples was described by the authors (57).

The procedure was readily adaptable to providing either relative or absolute fatty acid content of fats and oils but was especially convenient for routine analysis of large numbers of samples where only relative values were desired. The procedure described herein is a result of modifications of the former procedure but differs in the amount and kind of information obtained and the manipulations performed. Absolute quantitative determination of both fatty acid and glycerol content of fats and oils is realized. The fatty acids are determined as their methyl esters and glycerol as isopropylidene glycerol (IPG).

Absolute quantities (μmoles of component per 100 mg. oil analyzed) of both moieties of a fat or oil are obtained without making adjustments calculated from losses in the internal standard; recoveries (mg. fat by analysis per 100 mg. fat analyzed) are calculated directly from the GLC analyses. Internal standards are used only as indicators of faulty manipulations or incorrect standard curves. This report describes the modified procedure and data which establish the validity of its use are presented and discussed.

APPARATUS AND REAGENTS

Apparatus. GLC analyses were performed on an F and M model 500 linear-programmed-temperature gas chromatograph equipped with a Disc Instruments, Inc., disc integrator. Bristol 1 mvolt recorder, and a hot wire detector. Aluminum columns, 1/4 inch by 6 feet, packed with 14.5% EGS on Anakrom 100 to 110 mesh, type A, were used.

Reagents. EGS, ethylene glycol succinate (Applied Science Laboratories).

Anakrom, 100 to 110 mesh, type A (Analabs, Inc.).

Triglycerides, 99.5% purity (Mann Laboratories), used without further purification.

Methyl esters (Mann Laboratories), used without further purification.

Methanol, less than 0.02% water (British Drug Houses, Ltd.).

Benzene, reagent grade, dried over sodium.

2,2-Dimethoxypropane (Dow Chemical Company) redistilled from 76° to 79° C.

Methanolic HCl: Bubble dry HCl gas into dry methanol until it is at least 10% HCl (w./v.).

Methanolic sodium hydroxide: React clean strips of sodium metal in dry methanol until the solution is approximately 2.0N.

Solid neutralizer: Mix $NaHCO_3$, Na_2SO_4 and Na_2SO_4 in a 2:1:2 ratio by weight. The mixture is dried overnight at 110° C. and stored in an airtight container.

PROCEDURES

Transesterification of Triglyceride Mixtures and Fats and Oils for Accuracy and Precision Studies. Approximately 35 μmoles of each of several triglycerides were accurately weighed into a 25-ml. glass stoppered Erlenmeyer flask and reagents were added in the following order: 10.0 ml. benzene, 4.0 ml. DMP, 5.0 ml. methanol and 1.0 ml. of 2.0N sodium methoxide. The mixture was swirled, allowed to stand at room temperature for 5 minutes and sufficient methanolic HCl, determined by prior titration, added to supply about 0.3 mmoles of excess HCl. The mixture was swirled again and allowed to stand 50 minutes. About 1.5 grams of solid neutralizer was then added and the mixture swirled periodically during a 30 minute period. The mixture was allowed to settle and the supernatant decanted into a 25-ml. volumetric flask. The residue was washed twice with two 2-ml. portions of methanol which were also decanted into the volumetric flask. The volume was adjusted to 25 ml.

with methanol and 50-μl. aliquots were withdrawn for GLC analysis.

Fat and oil studies involved accurately weighing out approximately 200 mg. of the material and performing the analysis in the same way as that used for the triglycerides.

Time Course-of-Reaction Studies. Triglyceride mixtures and volumes of reagents used were the same for all time course-of-reaction studies as those described in the preceding section.

For the data in Figure 1, 2-ml. aliquots were periodically removed from the basic solutions, placed into 3-ml. volumetric flasks containing the exact amount of methanolic HCl needed to neutralize the aliquot, the volume adjusted to 3.0 ml. with methanol and 50-μl. aliquots taken by syringe for GLC analyses.

Data presented in Figure 2 were obtained by merely adding increments of HCl in the form of methanolic HCl, to the 3-ml. reaction mixtures described in the preceding paragraph. The acidified solutions were allowed to stand for 5 minutes and 0.25 gram solid neutralizing agent added. Estimations of the additional dilutions introduced by this procedure were made and 50-μl. aliquots analyzed by GLC.

Data presented in Figure 3 were obtained by acidifying the 20-ml. reaction mixtures with about 0.3 mmoles of excess HCl 5 minutes after addition of the sodium methoxide. Periodically, 2-ml. aliquots were removed from these reaction mixtures and transferred to 3-ml. volumetric flasks containing 0.25 gram solid neutralizer. The volume was adjusted to 3.0 ml. with methanol and 50-μl. aliquots chromatogrammed.

Conditions for GLC. Linear programmed temperature techniques were used to obtain resolution of IPG and low molecular weight esters. This involved making 50-μl. injections at 85° C. followed by a 3 minute

isothermal period and then programming at 11° per minute to 197° C. The latter temperature was maintained throughout the remainder of the chromatogram. Helium at a flow rate of 80 ml. per minute was used as the carrier gas.

RESULTS AND DISCUSSION

Reaction Conditions. Variation of the volume of 2.0N methanolic sodium methoxide used in the procedure from 1.0 ml. to 5.0 ml. had little effect on the formation of esters from triglyceride fatty acids. In all studies, the maximum yield of total esters (80%) was obtained within 5 minutes after adding sodium methoxide after which the total ester content decreased for a time and then increased to nearly 80% again. IPG formation was only about 40% complete and varied in the same manner as ester formation; these results are shown in Figure 1. However, incremental additions of HCl to these same reaction mixtures resulted in progressive increases in transesterification and IPG formation (Figure 2).

Data in Figures 1 and 2 clearly indicated that 5 minutes under basic conditions followed by very mildly acidic conditions would result in complete conversion of the triglycerides to IPG and the corresponding methyl esters in a short period of time. Previously, it had been determined that highly acidic conditions allowed only 60 to 70% conversion of glycerol to IPG even though transesterification was complete after 8 hours (57).

Figure 3 shows the time course-of-reaction study designed to determine the time necessary for complete transesterification and IPG formation under the acidic conditions prescribed in the procedure. The curves were extrapolated from 10 minutes to zero time (time of acidification) to

intersect the ordinate at points corresponding to those obtained after 5 minutes under basic conditions (Figure 1). Extrapolation was necessary because the course of reaction was not followed during the first 10 minutes after acidification. As a result of this study, a 50 minute period under the prescribed acidic conditions was chosen for the final procedure.

Precision and Accuracy Studies. Fatty acid contents of the oils listed in Table I are expressed on an absolute basis (μmoles ester per 100 mg. fat) and are averages of duplicate determinations. Standard deviations are also included and ranged from 0.00 to 9.06. For major components, such as palmitate (C_{16}), oleate ($C_{\overline{18}}$) and linoleate ($C_{\overline{\overline{18}}}$), the relative error ranged from about ±0.3 to ±3.0%. However, for minor components, such as myristate (C_{14}), stearate (C_{18}), linolenate ($C_{\overline{\overline{\overline{18}}}}$), arachidate ($C_{20}$), behenate ($C_{22}$) and lignocerate ($C_{24}$), the relative error ranged from 0% to about 30%. These larger errors were due mainly to instrumentation problems involved with peaks that were either small, diffuse or both. The disc integrator introduced as much as 10% error into sharp peaks below 10 units deflection on the chart and progressively greater error into peaks that were equally low and progressively more diffuse. Since all but two (C_{14} and $C_{\overline{18}}$) of the lesser components appeared in the latter part of the chromatograms where diffusion was most prominent, nearly all had a high relative error. Nevertheless, precision of measuring the total ester content was very satisfactory as evidenced by the average values and standard deviations listed in the first column in Table II. The relative error ranged from about ±0.1 to ±2.3%. Thus the data in Table I and the first column of Table II served to establish the lower and upper limits of precision in measuring absolute amounts of individual fatty acids as well as total fatty acids of a fat or oil.

Accuracy was evaluated in two different ways. Three different mixtures of tripalmitin, trimyristin, triolein and tristearin were analyzed using the prescribed procedure and the results along with mean values and standard deviations are presented in Table III. Analytically determined values for each ester and for glycerol averaged very nearly 100% of the amounts added to the reaction mixtures as triglycerides. The standard deviations ranged from ± 1.5 for oleate to ± 4.1 for myristate corresponding to relative errors of about ± 1.5 to $\pm 4.1\%$ respectively. These data leave little doubt that the glycerides were completely converted to IPG and their corresponding methyl esters. However, the inherent error is great enough to warrant performing analyses in triplicate.

Two criteria for testing the accuracy of analyses of fats and oils are inherent in this procedure: (1) the number of μmoles total ester by analysis should equal the μmoles of glycerol found by analysis multiplied by three. If equality exists, the sample is probably pure triglyceride and the preciseness of measurement of glycerol and fatty acid content is further verified. (2) Also, if equality exists and the sample is pure triglyceride, the sum of the weights of total esters and glycerol found by analysis minus the water of reaction should equal the weight of sample used for analysis. If so, the accuracy of the measurements is established. Application of these criteria to the data from the 9 fats and oils analyzed revealed agreement between glycerol (second column of Table III) and fatty acid contents (first column ot Table II) of the 9 fats and oils analyzed within limits of precision except for lard, soybean oil, corn oil and peanut oil. Of these, the fatty acid content of lard was a little low compared to glycerol indicating incomplete measurement of all the glyceride fatty acids present. However, fatty acid contents of the latter

3 oils were slightly higher indicating the presence of non-glyceride fatty acids in the oils or inaccurate measurement of glyceride fatty acids present. Additional information was provided by calculating for each fat and oil the weight of oil determined by analysis per 100 mg. sample analyzed. These values are listed in the third column of Table II. Recovery of lard was about 5% low indicating incomplete measurement of fatty acids present which was in agreement with the conclusion drawn by comparing the total fatty acid and glycerol contents. However, peanut oil and corn oil reciveries were both high indicating that the relatively high total ester content was probably the result of inaccurate measurement rather than the presence of non-glyceride fatty acids. Therefore, even though the precision, as indicated by the standard deviations, is within ±2%, the accuracy, as measured by percent recovery, is about ±3%. These data established the degree of purity of the fats and oils analyzed, the accuracy and precision of measuring the glycerol and fatty acid contents, and allowed the expression of these contents on absolute terms not dependent on estimations based on internal standards. The same criteria should be applicable to samples which are pure or nearly pure mono- or diglycerides.

Average molecular weights calculated from the amount of glycerol determined by analysis and the weight of sample analyzed are included in the fourth column of Table II along with standard deviations. Because they were calculated from glycerol content, these molecular weights were no more precise or accurate than duplicate values from which they were calculated. However, the confidence placed in these values should be greater than those determined from saponification numbers because of being able to first establish whether or not the sample is pure triglyceride.

Calculations. Some minor components present in trace amounts were

not included in Table I because their identities were not definitely established. Thus, tallow contained minute amounts of materials with retention times expected for tetradecenoate ($C_{\overline{14}}$), pentadecanoate (C_{15}), hexadecadienoate ($C_{\overline{\overline{16}}}$) and heptadecanoate ($C_{17}$) while lard contained a trace amount of material corresponding to a retention time expected for heptadecanoate (C_{17}). However, estimations were made for these trace components and included in values in the first and third columns of Table II.

Area-to-μmole relationships were obtained for each methyl ester and IPG (57) from the chromatograms of an appropriate mixture of standard methyl esters and IPG dissolved in methanol. These relationships were then used to calculate the amounts of fatty acids and glycerol in the fats, oils and triglyceride mixtures.

All samples analyzed contained the same amount of internal standard (methyl caprate). If the area of the internal standard changed by more than ±2%, injection of the standard solution of methyl esters was repeated and new unit areas calculated; however, this was necessary only occasionally. Increments from 10- to 50-μl. were chromatographed to insure linearity of response of the detector over the range of concentration being analyzed.

SUMMARY

A rapid, simple and quantitative method has been described for determination of the glycerol and fatty acid contents of fats and oils by gas-liquid chromatography. The method yields information that provides absolute criteria for reporting fat and oil composition not provided by other methods. Fatty acids are determined as their methyl esters and glycerol as isopropylidene glycerol. The latter compound serves as a

convenient marker for determining relative retention times.

ACKNOWLEDGEMENTS

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TABLE I

PRECISION STUDY: FATTY ACID CONTENT OF VARIOUS FATS AND OILS

	μmoles per 100 mg. and standard deviation									
	C_{14}	C_{16}	$C_{\overline{16}}$	C_{18}	$C_{\overline{18}}$	$C_{\overline{\overline{18}}}$	$C_{\overline{\overline{\overline{18}}}}$	C_{20}	C_{22}	C_{24}
Lard	4.9 ±0.50	83.7 ±0.35	7.9 ±0.42	35.4 ±1.68	141.5 ±1.91	43.2 ±0.78	4.7 ±0.50			
Palm Oil	4.6 ±0.77	161.5 ±3.46	0.4 ±0.04	13.9 ±0.10	127.2 ±3.36	36.0 ±0.92	2.8 ±0.00			
Tallow	12.3 ±0.36	85.3 ±0.50	8.3 ±0.36	74.0 ±3.68	130.9 ±1.42	5.8 ±0.36	3.5 ±0.05			
Cottonseed Oil	3.5 ±0.42	71.8 ±2.89	1.4 ±0.04	8.0 ±0.85	65.9 ±1.76	197.9 ±0.64				
Soybean Oil	0.6 ±0.07	36.8 ±0.10	0.2 ±0.00	13.4 ±0.10	85.5 ±2.12	171.9 ±2.96	26.4 ±1.77			
Hardened Soybean Oil		40.3 ±1.84		26.1 ±0.76	212.2 ±6.78	44.1 ±0.35	2.85 ±0.49			
Safflower Oil		25.1 ±0.10		7.8 ±0.00	47.6 ±0.92	262.4 ±9.06	1.7 ±0.06			
Corn Oil		41.7 ±0.85		6.1 ±0.00	94.6 ±0.99	189.9 ±0.64	3.5 ±0.05			
Peanut Oil		38.2 ±1.20		8.0 ±1.28	158.8 ±1.34	118.2 ±0.92	5.6 ±1.20	3.05 ±0.92	9.65 ±0.10	2.55 ±0.60

TABLE II

PRECISION AND ACCURACY STUDY WITH VARIOUS FATS AND OILS

	μmoles total fatty acid per 100 mg. fat	μmoles glycerol per 100 mg. fat X 3	total mg. fat determined per 100 mg. fat analyzed (% recovery)	average molecular weight
Lard	325.3±4.80	343.7±12.79	95.7±1.18	873.1±32.35
Palm Oil	346.2±4.52	347.8±11.73	100.6±0.74	862.8±24.26
Tallow	326.2±7.14	336.1± 0.85	96.6±0.84	893.1± 3.20
Cottonseed Oil	348.5±6.22	346.4± 6.23	103.7±1.51	865.9±15.52
Soybean Oil	334.8±6.01	319.4± 3.21	99.8±1.65	937.6± 9.55
Hardened Soybean Oil	325.5±6.24	326.0± 4.95	98.5±1.36	920.3±13.86
Safflower Oil	344.6±8.34	318.8± 4.88	103.1±2.52	940.7±14.45
Corn Oil	335.8±1.53	321.0± 5.09	103.1±1.44	934.7±14.98
Peanut Oil	343.2±0.32	329.0± 8.91	104.2±0.27	912.1±24.67

TABLE III

ACCURACY STUDY ON MIXTURES
OF PURE TRIGLYCERIDES

	% Recovery of Fatty Acids Added as Triglyceride				
Sample	C_{14}	C_{16}	C_{18}	$C_{\overline{18}}$	IPG
1	95	106	95	100	102
2	109	100	101	102	97
3	100	101	101	103	104
Mean	101.3	102.3	99.0	101.7	101.0
Std. Dev.	±4.10	±3.24	±3.54	±1.58	±3.67

Figure 1. Time course-of-reaction at room temperature using basic catalysis on a mixture of pure triglycerides

○ Mole percent transesterification of total fatty acids added as triglycerides

△ Mole percent conversion of glycerol to IPG added as triglycerides

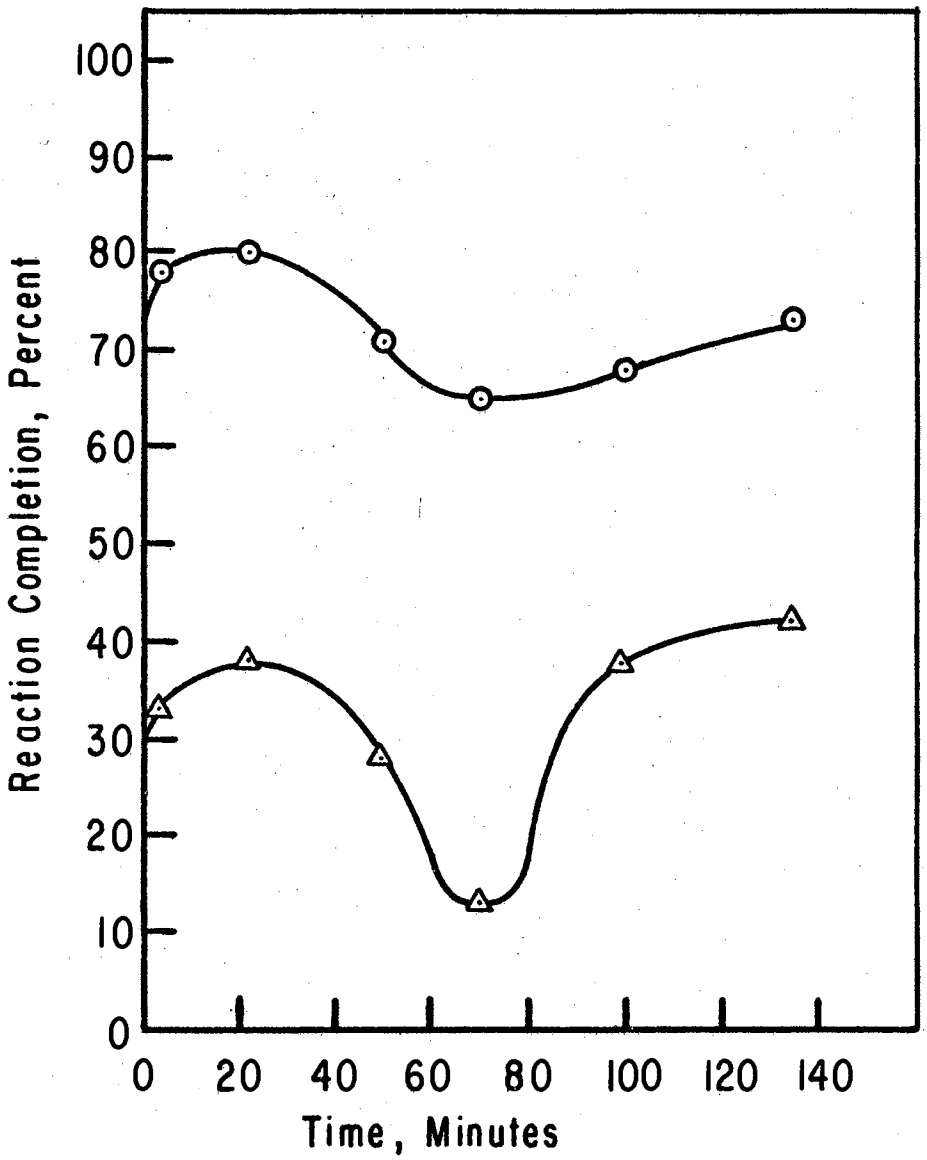

Reaction Completion, Percent
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
Time, Minutes

Figure 2. Effect of acid on mixtures of pure triglycerides that had already been subjected to basic conditions. Abscissa is mmoles HCl added to a 3 ml. reaction mixture

△ Mole percent transesterification of fatty acids added as triglycerides

○ Mole percent conversion to IPG of glycerol added as triglycerides

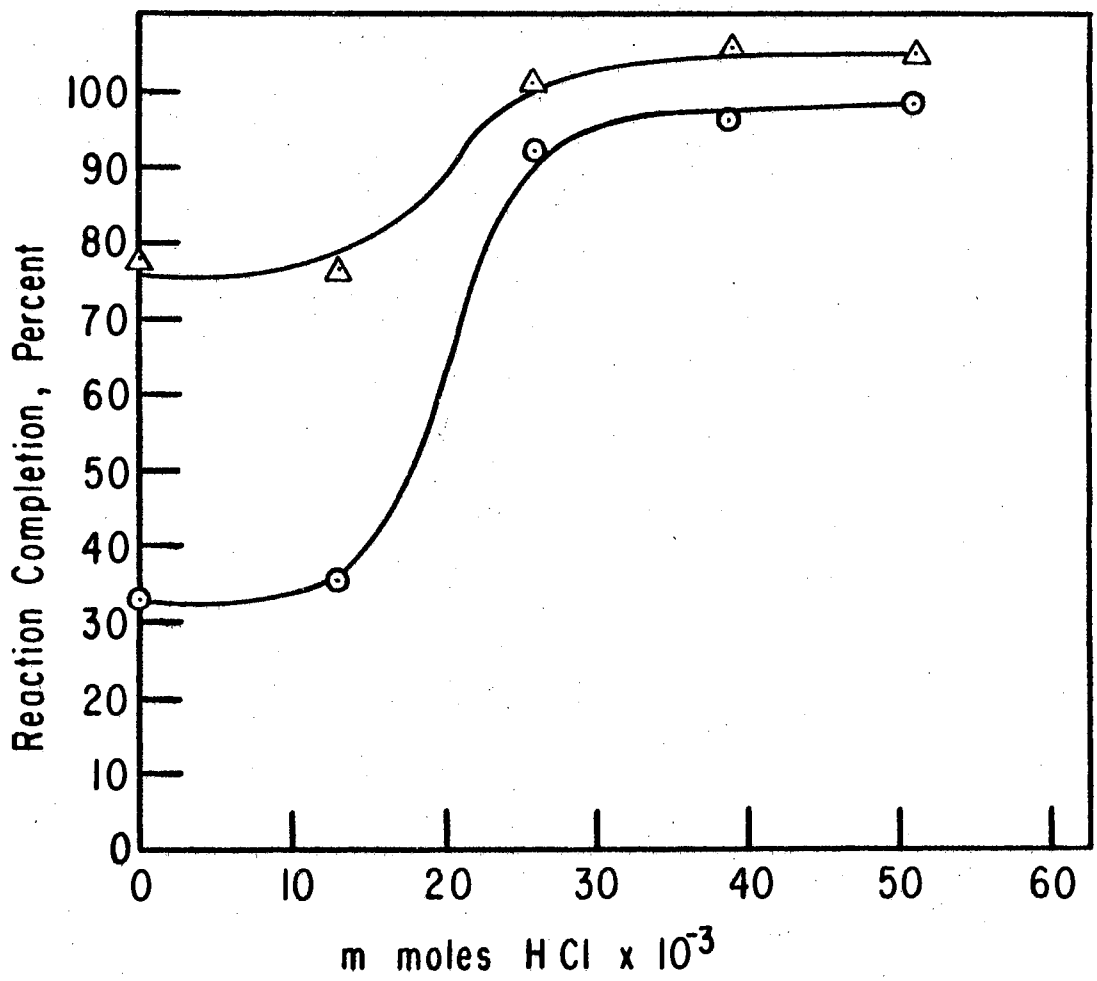
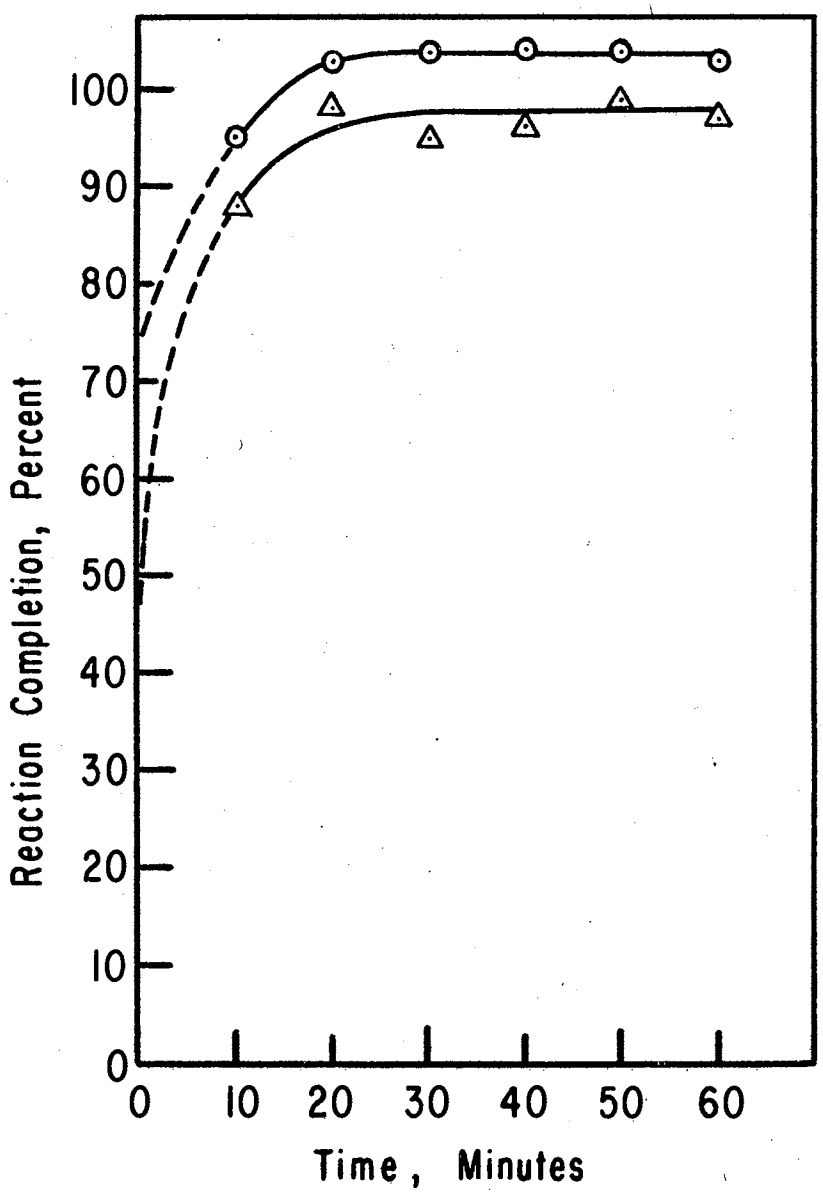

Reaction Completion, Percent
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
m moles H Cl x 10⁻³

Figure 3. Time course-of-reaction study on a mixture of pure triglycerides at room temperature to determine the time necessary for complete transesterification and IPG formation under acidic conditions (0.30 mmoles HCl per 20 ml. reaction mixture) after 5 minutes under basic conditions

○ Mole percent transesterification of total fatty acids added as triglycerides

△ Mole percent conversion to IPG of glycerol added as triglycerides


Reaction Completion, Percent
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Time, Minutes

CHAPTER V

PRECURSORS OF ROASTED PEANUT FLAVOR

Very little is presently known about the compounds responsible for the typical flavor of roasted peanuts or the precursors which give rise to flavor during roasting. At the time of a review by Hoffpauir (27), a few of the components of gases given off during roasting had been identified and a measurable decrease in total sugars during roasting had been noted (26). Gaseous components identified included derivatives of tetrahydrofuran, ammonia, hydrogen sulfide and diacetyl. The major gaseous component was found to be carbon dioxide. Also, Pickett and Holley had shown that mixtures of amino acids and carbohydrates reacted to produce tetrahydrofuran derivatives along with noticeable browning and discernable aromas. On the basis of the meager information available, Hoffpauir speculated on the precursors responsible for the formation of peanut flavor during roasting. The two main protein components of cotyledons, arachin and conarachin, which have been classified as reserve proteins (58), were implicated as precursors on the basis of their unusually high sulfur content. The implication was made to explain the presence of sulfide in roasting gases. Sucrose was implicated as a precursor on the basis of the loss of total sugars during roasting and on the basis of the appearance of tetrahydrofuran derivatives. Peanut cotyledons contain about 4.5% total disaccharides of which sucrose is the major component. Protein-bound and free amino

acids were implicated for similar reasons. Since 1953, nothing in the literature has added significantly to this sketchy picture.

Work reported here represents initial results of an integrated program to improve peanut quality by identifying the precursors which give rise to typical peanut flavor during roasting.

APPARATUS AND REAGENTS

Apparatus. Roasting studies and roasting of isolated fractions were performed in an electrically heated rotisserie fitted with a cylindrical wire basket designed specifically for this purpose.

In preparing cotyledons, hearts and seed coats were removed by hand, but the cotyledons were separated on a laboratory splitter.

For gel filtration studies, cotyledons were ground in a Quaker mill, model 4E, and the oil extracted with a large Soxhlet extractor using n-hexane. For particulate fractionations, cotyledons were disintegrated in an Serval Omnimixer fitted with a 400 ml. stainless steel cup.

Centrifugations were carried out in a Lourdes, model LR, centrifuge using a 9 inch rotor and 250 ml. bottles. Specific gravity adjustments were made with a Westphal balance.

Fractions from gel filtrations were collected on an automatic fraction collector and the elution pattern studied by scanning each fraction between 400 and 220 mμ using a Cary, model 14, recording spectrophotometer.

Drying of gel filtration fractions was done by lyophilization.

Photomicrographs of the particulate fractions were taken using a Unitron, Bi-1796, with a Leica III-C camera attachment. Cursory

examinations were performed with a Bausch and Lomb microscope at magnification of 900 X.

Reagents. Optical Hexane was prepared by distilling high purity n-hexane over KOH pellets onto a silica gel column. The hexane collected from the column lacked absorbtion in the 230 to 260 mμ range compared to air.

Sephadex gels, obtained from Pharmacia, Uppsala, Sweden, were swollen, washed and packed on columns according to the procedures outlined by Fasold (59).

Refined cottonseed oil was obtained from the Great Western Foods Company, Fort Worth, Texas.

Carbon tetrachloride, reagent grade, was used for adjustment of the specific gravity of the cottonseed oil.

Three varieties of Spanish peanuts, Starr, Spantex and Argentine, were used in the roasting studies. However, Argentine were used exclusively for the gel filtration and particulate fractionation studies.

Spray reagents used for detection of spots on paper chromatograms: 1. 0.2% ninhydrin in water-saturated n-butanol, 2. equal volumes of 0.1N silver nitrate and 5N ammonium hydroxide and 3. 0.04 % bromocresol green in 95% ethanol. Chromatograms were developed on Whatman number 1 paper in n-butanol:acetic acid:water (8:2:2) solvent.

PROCEDURES

Roasting Studies. Roasting data were obtained by placing 28 grams of raw peanuts in the roaster which was preheated to the desired temperature. Heating was continued until roasting was complete as judged by color and taste. If an under- or over-roast was indicated, the

process was repeated until a satisfactory roast was obtained. In one case, peanuts of Starr variety were roasted by placing them in the roaster at room temperature and increasing the temperature to the desired level as rapidly as possible. Logarithms of the time necessary for complete roasting were plotted versus the inverse of temperatures used.

Roasting Individual Fractions From Fractionation Studies. Individual fractions obtained from the gel filtration and particulate fractionation experiments were macerated with solvent extracted peanut oil, wrapped in aluminum foil and roasted at 400° F. Since the samples could not be observed, several roastings were performed on each fraction before a satisfactory roast was obtained, as judged by color and aroma, and taste where possible.

Precursor Isolations Using Gel Filtration. The flow diagram in Figure 1 briefly outlines the procedure for separation of flavor precursors using this technique. Fifty grams of the dry, fat-free, finely ground cotyledons were extracted with 500 ml. of 1M NaCl for 3 hours at 4° C. Suspension was maintained by gentle stirring on a magnetic stirrer insulated from the beaker by an asbestos pad. Next, the suspension was filtered through a plug of glass wool and the filtrate centrifuged at 8000 X g for 30 minutes at 4° C. and the supernatant decanted. Two-hundred ml. of this solution was placed on a 2-liter Sephadex G-25 column in 1M NaCl and developed with 1M NaCl at 100 to 200 ml. per hour. Aliquots of each 12 ml. fraction collected were diluted when necessary and scanned to observe the magnitude of peak absorbancies and shifts in wavelength of maximum absorbancies that occurred when the composition of absorbing material in the eluant changed. In this way

optical density per ml. at maximum absorbancies of the eluate was calculated and plotted versus volume of eluant to provide an elution pattern. This is shown in Figure 2 along with the wavelength of maximum absorbance of each peak. Contents of tubes lying between the minima on the curves of the elution pattern were combined, assigned fraction numbers (Figure 2), lyophilized and the resultant solid-salt mixtures roasted.

Aliquots were taken from alternate tubes in each fraction for paper chromatography before contents of the tubes were combined.

An alternate fractionation procedure used involved adjusting the saline solution obtained after centrifugation to pH 2.5 using 2N HCl. The resulting heavy white precipitate was centrifuged at 8000 X g after standing at 4° C. overnight. The supernatant was adjusted to pH 6.3 and filtered using 1M NaCl on a 2-liter column of Sephadex G-25 in 1M NaCl or alternatively on the same column recycled into the water form using water as the eluant, for separation of components which did not precipitate at pH 2.5.

Precursor Localization by Particulate Fractionation. Figure 3 contains a flow chart outlining the major steps involved in this nonaqueous, density gradient centrifugation procedure. With the variety of peanuts used in our laboratory, the density gradient centrifugation procedure outlined by Dieckert et al. (51) resulted in the aleurone grains, protein bodies and starch grains sedimenting in the same fraction. Consequently, specific gravity adjustments other than those used by Dieckert were made in an attempt to effect a similar separation.

Peanut cotyledons were prepared in the same manner as for the gel filtration experiments. One hundred and fifty grams of the cotyledons were homogenized for 2 minutes in 300 ml. of oil. This was done in

three separate batches, 50 grams at a time with 100 ml. of cottonseed oil. The thick brei were combined and centrifuged at 300 X g for 5 minutes. The supernatant was then decanted, the centrifugate resuspended in 300 ml. of oil and re-centrifuged at 300 X g for 2 minutes. Microscopic examinations indicated the combined centrifugate was mostly vascular tissue with some particulate material remaining. The supernatants were combined and centrifuged at 2600 X g for 15 minutes leaving most of the reticular material in suspension. At this point the centrifugate was composed mostly of the three particulate bodies: aleurone grains, protein bodies and starch grains. Some needle-like bodies were also present. When the centrifugate was resuspended in oil, adjusted to a specific gravity of 1.461 and centrifuged at 2600 X g for 30 minutes, three centrifugates and a clear oil medium resulted. The layer floating on the oil (top layer) was removed with a spatula and the oil decanted. A second layer adhering to the side of the bottle (middle layer) was next removed with the aid of a large rubber policeman and, finally, the bottom layer was removed in a similar manner.

All the fractions except the reticular suspension were washed free of oil on filter paper with carbon tetrachloride, dried at 40° C., and stored in a freezer. Each fraction was suspended in oil, examined under the microscope, heated to 180° C. for periods of 5, 10 and 15 minutes, and re-examined. Also, these fractions were roasted in the rotisserie in the usual manner for taste, color and aroma observations.

RESULTS AND DISCUSSION

Significance of Roasting Studies. Considering what has been

learned of the morphology of the parenchymal cells of cotyledons (53), these cells represent peculiar reaction vessels. Thinking in these terms prompted us to classify the chemical and physical events leading to the formation of flavor components into one or both of two categories:

1. Intermolecular reactions between different types of molecules or between molecules of the same type in which close proximity of other molecules is essential. Example: condensation between an amino acid and carbohydrate or between two molecules of amino acid.

2. Intramolecular reactions in which the close proximity of another molecule is not necessary. Such reactions as decarbonylations, decarboxylations, water eliminations and intramolecular rearrangements would fall into this category.

Some generalizations could be made immediately. Considering the first of the above categories, it was evident that reactions of this type would be functions of both energy supply and collision frequency and would not be favored by compartmentalized, non-fluid media. No matter how great the energy supply, the proper reactions could not take place if collision frequency were very low or nonexistent. Concerning reactions of the second category, flavor formation would depend only on supplying sufficient energy to cause the necessary intramolecular conversions. Accordingly, this category predetermines the presence of flavor precursors in sufficient quantities; whereas, the first category would allow for the formation of precursors during the early stages of the heating process. Reactions in the second category would be favored by compartmentalized, non-fluid media. Data from roasting studies presented in Figure 4 established some points relative to this subject and were indicative of others. The critical minimum temperature for

flavor formation is close to 270° F. since the upper curve in Figure 4 approaches asymptotically a line perpendicular to a 1/T value corresponding to 270° F. Several attempts to roast several varieties of peanuts below this temperature failed. We concluded that temperatures above 270° F. must be maintained in order to develop typical roasted peanut flavor. This agrees closely to a minimum temperature of 120° C. reported by Pickett and Holley (26). Seemingly, temperatures of this order exclude the possibility of enzymatically catalyzed reactions giving rise to flavor components. Also, the linearity of the semilogarithmic plots for all three varieties in the 300° to 425° F. range suggested that the formation of flavor components was a function of available energy only. Stating this in another way, concentration and distribution of flavor precursors was such that activation energy became the rate limiting factor in the formation of flavor components. Furthermore, if this tenet were to hold, the semilogarithmic plots of the same variety of peanuts roasted at different rates of heating should intersect at the point where roasting was no longer a function of time, i.e., at that temperature where flash roasting would theoretically occur. This would be at a point where the logarithm of time equaled 0 in Figure 4. The two curves for Starr variety in Figure 4 represent two different rates of heating to the same final temperatures. Extrapolation of the two curves to zero time showed intersection did indeed occur. Apparently, in the region of linearity, the formation of flavor components was solely a function of energy.

These observations strongly suggested that flavor formation was predominantly an intramolecular process and would fall in category 2. However, the possibility of intermolecular reactions occurring was not

completely ruled out since all necessary molecular species could be strategically situated within the same compartment and sterically positioned such that collision might readily occur. Nevertheless, in light of the highly compartmentalized, highly dehydrated, quiescent state of mature cotyledons, this possibility seems rather remote unless the precursors are leached into the oil droplets surrounding the aleurone grains (53) becoming mobile enough to undergo the necessary intermolecular combinations for flavor formation. Additional light is shed on this subject by particulate fractionation studies.

The importance of this information became more relevent when the implications of intra- versus intermolecularity upon precursor isolation studies were considered. If intermolecularity were the rule, one could test for the success of any precursor fractionation procedure only by recombination for development of typical aroma and taste during roasting. On the other hand, if intramolecularity were the rule, one could inspect individual fractions for their particular contribution to typical flavor without numerous recombinations. Consequently, one could expect reasonable success with a precursor fractionation procedure which destroyed the integrity of cells if intramolecularity were the case. Such a procedure, involving gel filtration was undertaken.

Isolation of Flavor Precursors Using Gel Filtration. In view of the implication that proteins might serve as flavor precursors, a procedure was formulated which would provide the separation of the two main globulins, arachin and conarachin, (60) of cotyledon parenchymal cells as well as other cellular components. Saline solutions have been used to solubilize globulins and Naismith and McDavid (50) have successfully used 1M salt solutions to extract peanut seed globulins for ultra-

centrifugal studies; thus, 1M solutions were used for this procedure. Sephadex G-75 was first chosen on the basis that arachin was large enough to be totally excluded from the gel thus appearing at the eluting front. Conarachin would be close to the borderline of size for exclusion and would undergo some partitioning with the gel internal phase. Two very large peaks were observed in the elution patterns in the positions expected. One contained the largest amount of protein material on a nitrogen basis and appeared at the eluting front while the other appeared immediately behind it with a partition coefficient (K) equal to 0.6. Roasting results indicated that neither fraction contributed to typical peanut aroma or taste. Consequently, Sephadex G-25 was used for subsequent fractionations in which the two protein fractions appeared as a single peak at the eluting front (Figure 2). This choice allowed a cleaner separation of smaller components having K values greater than 0.

Salt extraction solubilized an average of 45% of the total solids including the flavor precursors. The insoluble residue from several extractions failed to contribute to typical peanut aroma or flavor when roasted. Components responsible for typical roasted peanut aroma were found in fractions 3 and 4 (Figure 2) which had maximum absorbancies at 258 and 262 mμ, respectively. Other fractions did not contribute to flavor in any of the many separations made. Complete desalting of these two fractions was never achieved because these compounds have K values very near that of NaCl (K=1). Consequently, roasting studies were done on the dry salt-solid mixtures making any taste observations nearly meaningless. However, a very marked browning consistently occurred only in the fractions which developed typical peanut aroma. This browning progressed into a deep chocolate color as roasting continued without developing a charred

aroma. This marked color change was considered to be a property of flavor development and an excellent marker for following flavor precursor isolation.

Paper chromatograms revealed the presence of two ninhydrin positive spots, one remaining near the origin, and one moving with an R_f of 0.10. A highly basic spot (R_f 0.19) which reacted yellow to ninhydrin was also present. Although the same three components were present in both fractions 2 and 3, most of the ninhydrin positive material was in the peak absorbing at 258 mμ. The ammoniacal silver nitrate test for sugars was negative in all chromatograms.

When the alternate procedure involving pH adjustment before gel filtration was used, about 95% of the protein material in fraction 1 (λ max. = 272) was precipitated and the only other absorbing materials remaining in solution were those absorbing at 258 to 262 mμ and at 310 mμ. Approximately 34% of the soluble material was precipitated leaving in solution about 32% of the weight of original fat free solids extracted. This protein precipitate was 15.7% nitrogen by Kjeldahl analysis and was devoid of any aroma or flavor when roasted. On the other hand, the supernatant maintained typical aroma characteristics after drying and roasting. When this supernatant was filtered on a 2-liter Sephadex G-25 column in water, the material absorbing at 258 to 262 mμ was eluted between 1296 and 1946 ml. as a suspension. The suspension was allowed to settle, the supernatants decanted and the precipitates combinded and roasted. No typical aroma or taste was observed in the precipitate but the combined supernatants did develop typical aroma and taste. The profound tendency for browning was still present. This supernatant still contained some salt which made the taste observations

difficult to interpret.

Localization of Flavor Precursor. The observation by Woodroof and Leahy (53) that oil pressed from peanuts contained aleurone grains, among other particulates, coupled with our observation that the settlings from oil pressed from freshly roasted peanuts were extremely potent carriers of peanut aroma led to this study of the particulates. Although all fractions roasted produced some typical peanut aroma and taste, the upper layer (aleurone grains and protein bodies) was by far the most potent in aroma and tasted very much like peanut butter. Examination of these fractions under the microscope before and after heating revealed that the protein bodies and aleurone grains maintained their gross structural integrity throughout the heating process (180° C.) which was comparable to normal roasting temperatures (Figure 5). Heating caused clumping of particulates and lightening of the inclusion bodies but the latter observation was not obvious from the photomicrographs in Figure 5. The starch grains (lower fraction) ruptured during heating producing small donut like holes in the center of the grain (Figure 6b) and an effervescence in the medium which was apparently due to the release of water vapor. This effervescence began at about 110° C. That starch grains were the only particulates which ruptured was shown by examining particulates for typical birefringence shown only by starch grains under cross polarized light. Also, the use of cross polarized light showed that starch grains were absent in the upper layer, appeared only occasionally in the middle layer and made up about 80% of the total bodies in the bottom layer. Some large protein bodies and aleurone grains as well as vascular and reticular materials were also present in the bottom layer (Figure 6). The distinction between

aleurone grains and protein bodies was made on the basis of the presence or absence of inclusion bodies within the particulates. On this basis, it appeared that the upper layer was mostly aleurone grains (note only three particles in Figure 5a were lacking inclusion bodies), whereas the middle layer contained mostly translucent spheres believed to be protein bodies (Figure 7). Weights of particulates obtained from each of the three layers as well as the weight of vascular material are included in Figure 3.

These data established the protein-body aleurone grain fraction as the specific location of flavor precursors and implicated the aleurone grains more strongly than the protein bodies. However, this choice cannot be made until a good separation of these two bodies is effected as judged by chemical analysis as well as the presence of inclusion bodies. Chemically, the two differ in that aleurone grains have a much higher ash and phytic acid content (51).

Using this procedure to obtain a fraction rich in precursors followed by gel filtration for separation and purification of individual components promises to provide sufficient precursor material for the elucidation of structures.

SUMMARY

The techniques used for these studies have revealed useful information concerning the locale and molecular origin of flavor precursors within peanut cotyledons. Several conclusions drawn concerning the molecular origin of flavor during roasting are: (1) Flavor does not appear to arise from the large globulin proteins and (2) it does not appear to arise from carbohydrate material per se. (3) Flavor does

arise from one or a combination of ninhydrin positive and basic compounds.

Apparently, flavor originates from rather specific types of micromolecules rather than the general macromolecular cellular components such as the large globulin proteins and the starches.

ACKNOWLEDGEMENTS

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Figure 1. Flow chart showing procedure for separation of flavor precursors from mature peanut cotyledons using gel filtration techniques

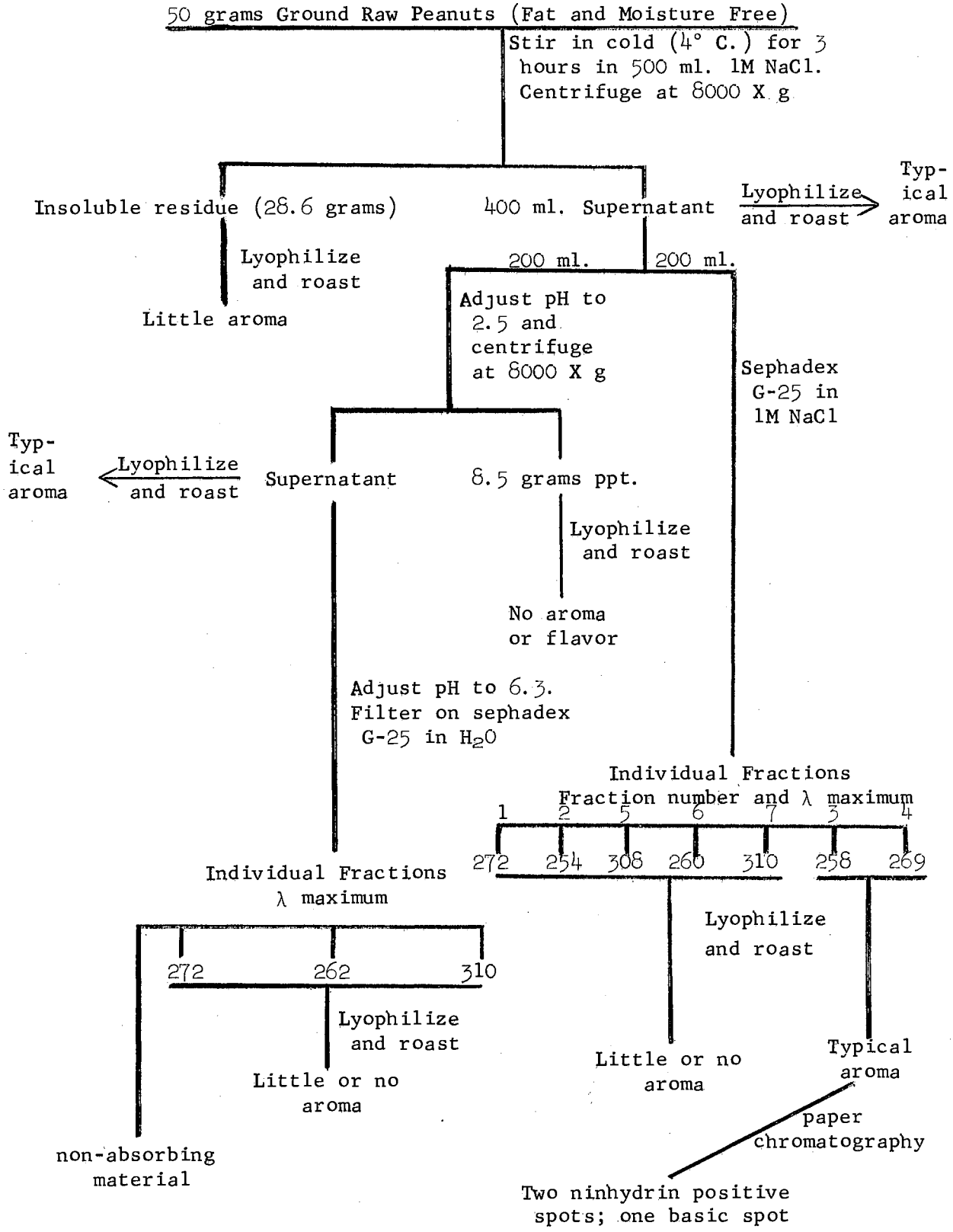

50 grams Ground Raw Peanuts (Fat and Moisture Free)
Stir in cold (4° C.) for 3 hours in 500 ml. 1M NaCl. Centrifuge at 8000 X g
Insoluble residue (28.6 grams)
400 ml. Supernatant
Lyophilize and roast
Typical aroma
Lyophilize and roast
Little aroma
200 ml.
200 ml.
Adjust pH to 2.5 and centrifuge at 8000 X g
Sephadex G-25 in 1M NaCl
Typical aroma
Lyophilize and roast
Supernatant
8.5 grams ppt.
Lyophilize and roast
No aroma or flavor
Adjust pH to 6.3. Filter on sephadex G-25 in H_2O
Individual Fractions
Fraction number and λ maximum
1 2 5 6 7 3 4
272 254 308 260 310 258 269
Individual Fractions
λ maximum
272 262 310
Lyophilize and roast
Little or no aroma
non-absorbing material
Lyophilize and roast
Little or no aroma
Typical aroma
paper chromatography
Two ninhydrin positive spots; one basic spot

Figure 2. Elution pattern of saline extract of peanut cotyledons from Sephadex G-25 column in 1M NaCl having the following characteristics:

Total Volume (V_t): 2010 ml.

Void Volume (V_o): 650 ml.

Internal Volume (V_i): 960 ml.

Flow Rate: 200 ml. per hour

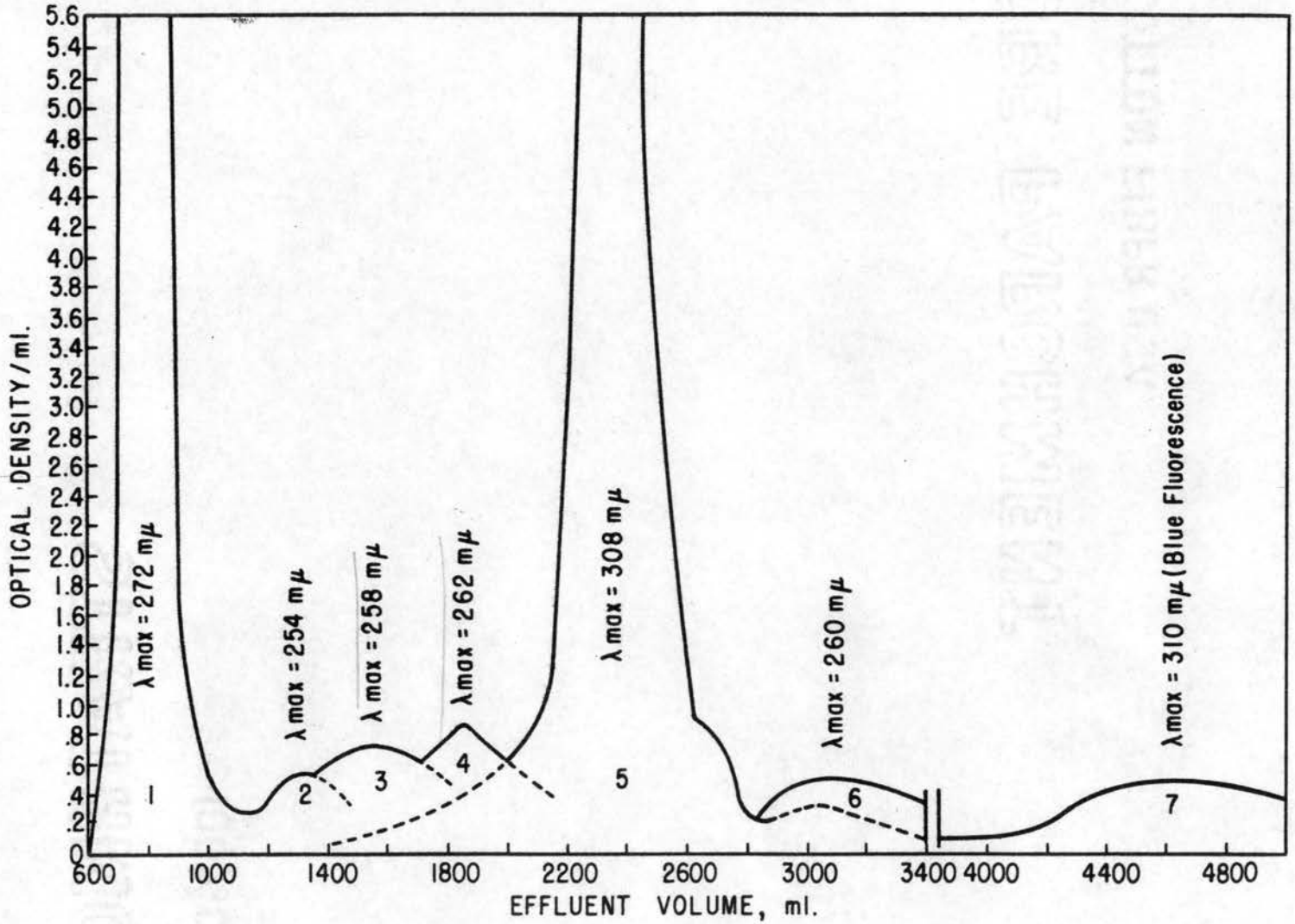

OPTICAL DENSITY/ml.
5.6
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
λ max = 272 mμ
λ max = 254 mμ
λ max = 258 mμ
λ max = 262 mμ
λ max = 308 mμ
λ max = 260 mμ
λ max = 310 mμ (Blue Fluorescence)
1
2
3
4
5
6
7
600
1000
1400
1800
2200
2600
3000
3400
4000
4400
4800
EFFLUENT VOLUME, ml.

Figure 3. Flow diagram showing procedure for fractionation of particulates from mature peanut cotyledons

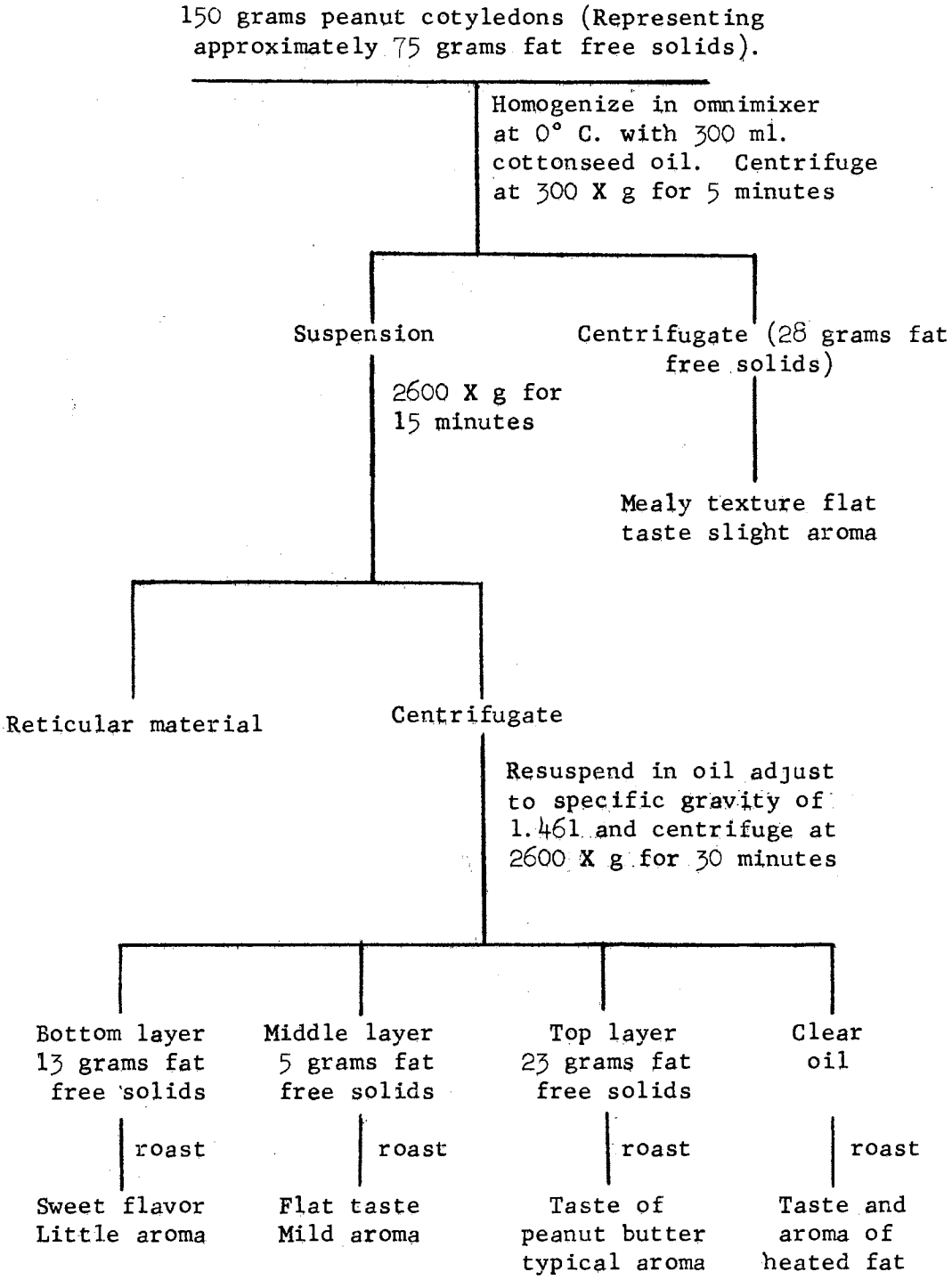

150 grams peanut cotyledons (Representing approximately 75 grams fat free solids).
Homogenize in omnimixer at 0° C. with 300 ml. cottonseed oil. Centrifuge at 300 X g for 5 minutes
Suspension
Centrifugate (28 grams fat free solids)
2600 X g for 15 minutes
Mealy texture flat taste slight aroma
Reticular material
Centrifugate
Resuspend in oil adjust to specific gravity of 1.461 and centrifuge at 2600 X g for 30 minutes
Bottom layer 13 grams fat free solids
Middle layer 5 grams fat free solids
Top layer 23 grams fat free solids
Clear oil
roast
roast
roast
roast
Sweet flavor Little aroma
Flat taste Mild aroma
Taste of peanut butter typical aroma
Taste and aroma of heated fat

Figure 4. Roasting studies on three peanut varieties: Plot of the logarithm of the time necessary to roast 28 grams of mature peanuts versus the inverse of the temperature used

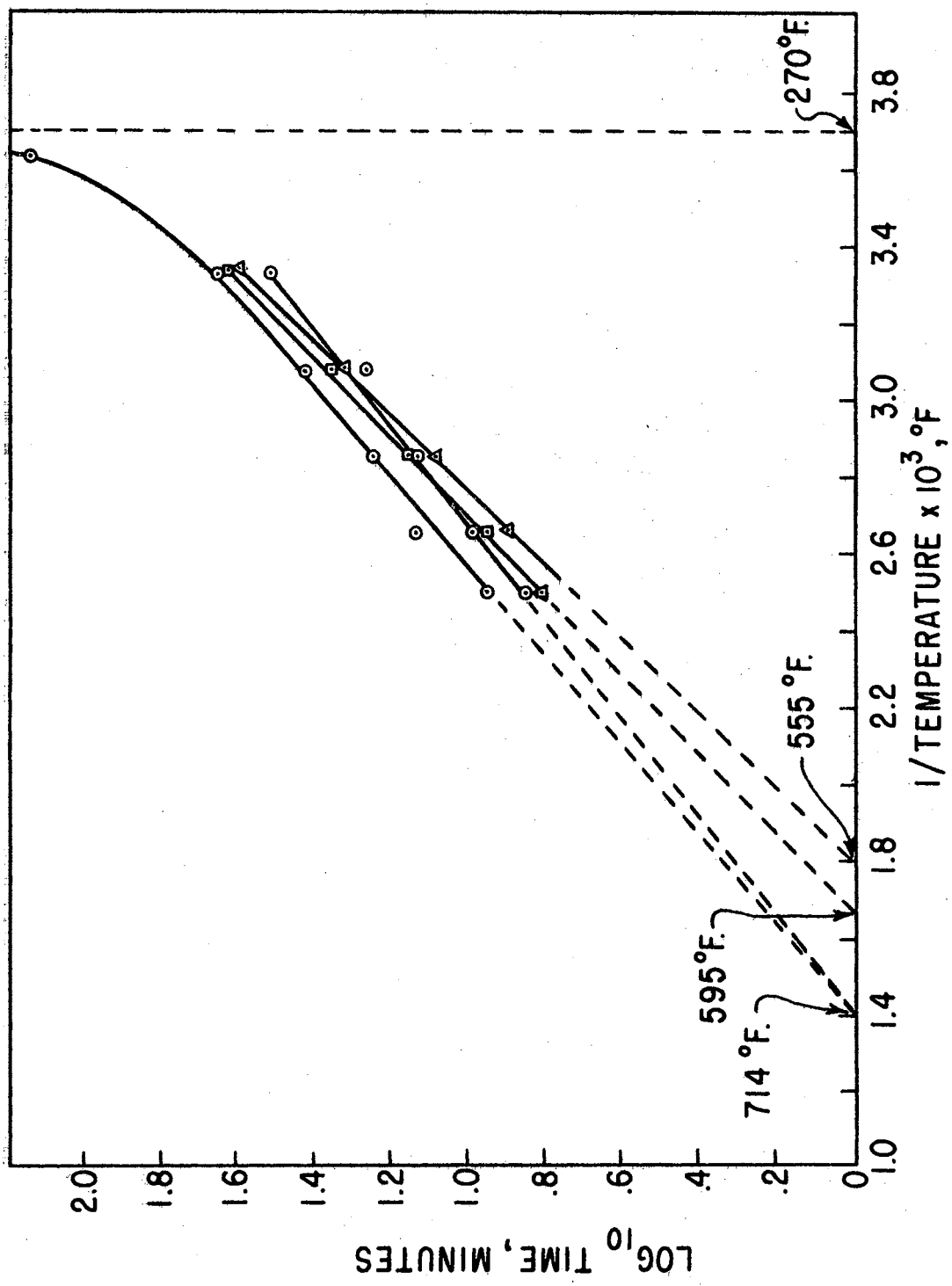
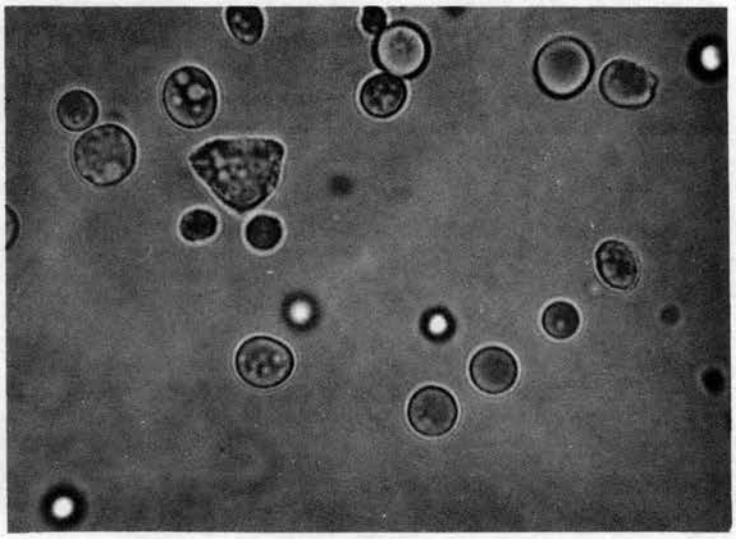

270°F.
555 °F.
595°F.
714 °F.
1.0
1.4
1.8
2.2
2.6
3.0
3.4
3.8
1/TEMPERATURE x 10³, °F
0
.2
.4
.6
.8
1.0
1.2
1.4
1.6
1.8
2.0
LOG10 TIME, MINUTES

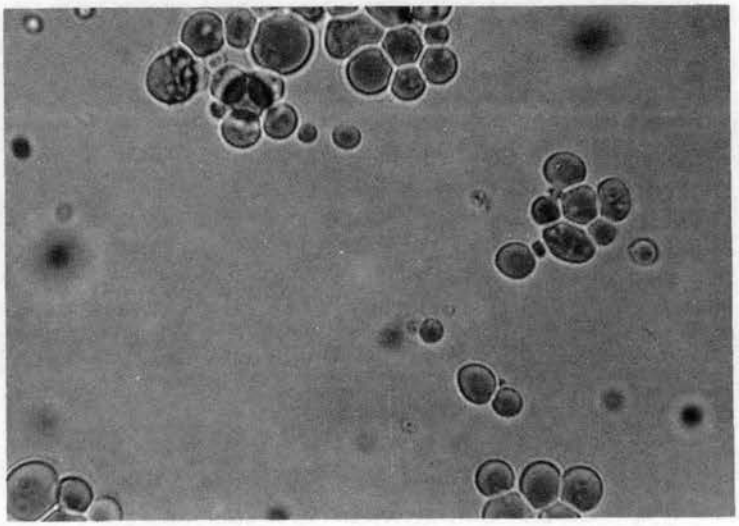
Figure 5. Photomicrographs of aleurone grains and protein bodies (top layer), magnification 1200X, isolated according to the prescribed procedure

a. Before heating, suspended in peanut oil

b. After heating 30 minutes at 180° C., suspended in oil, showing clumping



5a

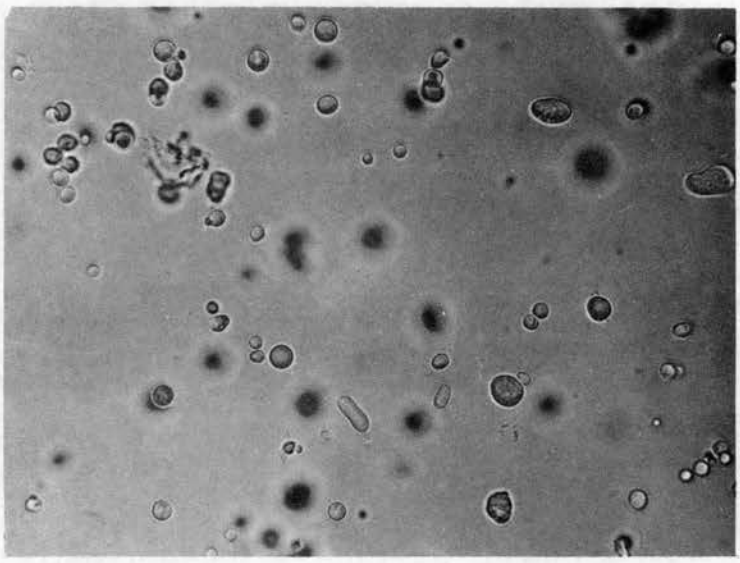


5b

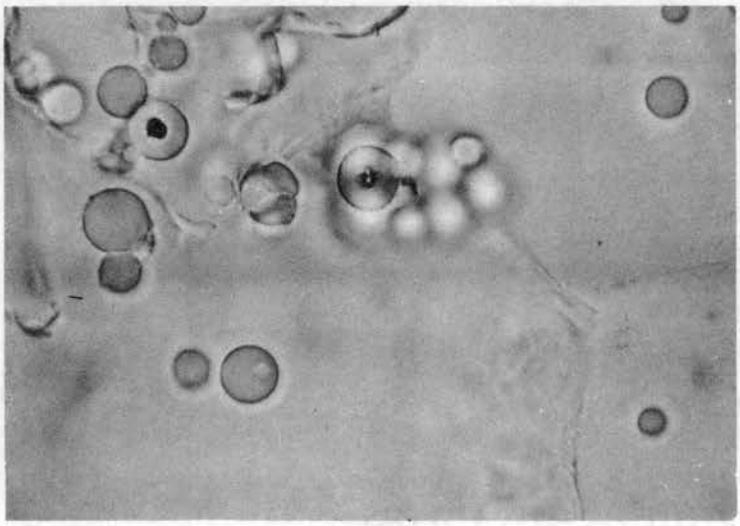
Figure 6. Photomicrographs of starch grains (bottom layer) isolated according to the prescribed procedure

a. Before heating, suspended in oil, magnification of 520X

b. After heating, suspended in oil, magnification of 1200X, showing donut-like holes in two of the starch grains

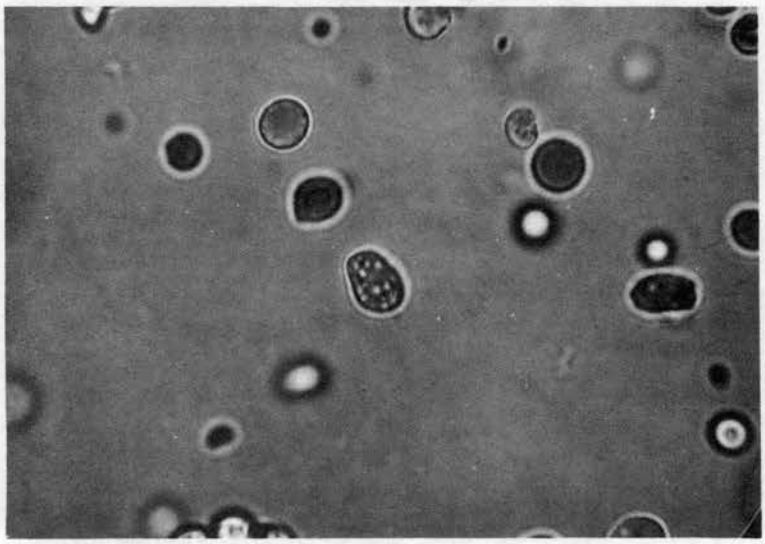


6a



6b

Figure 7. Photomicrograph of middle layer separated according to the prescribed procedure showing mostly protein bodies with some aleurone grains intermixed



CHAPTER VI

ISOLATION AND IDENTIFICATION OF FLAVOR COMPONENTS

At present, there are no published reports which identify the compounds responsible for the typical taste and aroma of roasted peanuts. The roasting studies of Pickett and Holley (26) showed that carbon dioxide accounted for 98% of the volatile substances given off during roasting; sulfides, ammonia and diacetyl were also identified. No attempt was made to determine to what extent, if any, these compounds contributed to typical roasted peanut flavor.

Knowledge of the structures of compounds responsible for typical flavor should precede the formulation of a research program designed to improve the flavor of roasted peanuts and peanut products. For example, preservation of the flavor of peanuts and peanut butter during storage may be best attained by first knowing what functional groups must be protected from losses due to oxidation, evaporation and other chemical and physical processes. Equally important, knowledge of structures of flavor components would help in predicting the structures of flavor precursors. Consequently, this work was initiated in hopes of identifying the compounds responsible for typical roasted peanut flavor.

Appropriately, interest in the chemistry of peanut flavor has developed at Oklahoma State University. Oklahoma is one of the major peanut producing states. It ranks fifth in acerage and sixth in production in the United States. In 1962 the 118,000 acres harvested in

Oklahoma produced 165,200,000 pounds of farmers' stock peanuts for an average yield of 1,400 pounds per acre (61).

APPARATUS AND REAGENTS

Apparatus. A vacuum degassing apparatus similar to the one used by Merritt et al. (39) to be used in the isolation and fractionation of volatile flavor components in peanut butter and on the oil pressed from roasted peanuts was constructed. This apparatus consisted of a Welch dual stage vacuum pump rated at 0.1 microns and 140 liters per minute, a three stage oil diffusion pump to obtain high vacuum, and a manifold connected to a U tube trap as shown in Figure 1. Several bulbs made from glass tubing of various sizes were used for performing "bulb to bulb" distillations on the manifold. Figure 1 also contains a schematic drawing of one of these tubes.

Degassing of the oil was accomplished by using a glass tower (Figure 2) consisting of a series of expansion bellows on 44 mm. tubing fitted with 250 ml. bulbs at each end of the tube to hold the oil. Constrictions of 1 to 2 mm. made in each bulb insured slow passage of oil onto the expansion bellows. These were jacketed to allow only about 14 mm. clearance (Figure 2) for water circulation. In this way, water of the desired temperature could be pumped rapidly through the jacket without introducing undue strain on the manifold because of the weight of water. This tower was attached perpendicularly to the manifold at the position shown in Figure 1 and rotated through 180 degrees periodically to renew oil flow from one end to the other.

One port of the manifold was used for the attachment of a cold cathode tube for vacuum measurements. All other ports were used for low

temperature "bulb to bulb" distillations.

Liquid nitrogen was used for -196° C. baths while the -140° C. baths were prepared according to the procedures of Sanderson (62). Dry-ice and trichloroethylene were used to provide temperatures of -80° C.

Steam distillations of peanut butter were performed from a 12 liter, two neck flask. One of these necks held a sleeve type stirrer attached to a cone-drive motor. To the second neck was attached one arm of a continuous extractor (Figure 3) fitted with a reflux condenser. The other arm was connected to a 500-ml. round bottomed flask containing 300 ml. of a hexane-ethyl ether (9:1 v./v.) mixture.

An ice-cooled (0° C.) rotary evaporator attached to a water aspirator was used to remove the solvents from the steam distillates.

Preparative gas chromatography of the steam distillates was performed on an F and M model 500, linear programmed-temperature gas chromatograph using 3/8 inch by 8 foot aluminum columns of 20% DEGA on Anakrom 100 to 110 mesh, type A, and 20% Apiezon L on Chromosorb W, acid washed. Helium was used as the carrier gas for both columns at a flow rate of 80 to 90 ml. per minute. Emerging compounds were trapped in tubes specifically designed for this apparatus (Figure 4) using -80° C. baths as coolants. Preparative gas chromatography of samples obtained from the degassing apparatus was accomplished using a flame ionization detector and splitting the effluent gas with a needle-valve allowing only about 10% of the effluent gas to flow through the flame. The remaining 90% passed through U tubles sealed with rubber septums (serum caps) and cooled in liquid nitrogen. A Hamilton fraction collector was used to aid in collecting individual fractions. The inlet system shown in Figure 5 was used to introduce the gaseous sample onto the column. The volume of sample intro-

introduced could be varied by merely replacing the 100 ml. chamber with a chamber of different volume. The sample was introduced onto the column by evacuating the inlet system, isolating it from the vacuum pump, opening it to the sample bulb, allowing the system to come to equilibrium as noted from the manometer, isolating it from the sample bulb, and finally opening it to the flow of carrier gas leading to the column.

Mass spectra were obtained on the CEC-21 model 103C mass spectrometer located in the research laboratories of the Continental Oil Company, Ponca City, Oklahoma. NMR (nuclear magnetic resonance) spectra were obtained with a Varian model A-60 spectrometer. Infra-red spectra were obtained with a Beckman model IR-5 spectrometer.

PROCEDURES

Degassing Techniques. The degassing system was first evacuated to 10^{-4} mm. of Hg and then isolated from the vacuum source by properly adjusting the T bore stopcock. While under vacuum, approximately 250 ml. of clear oil extracted from freshly roasted peanuts was introduced slowly into the lower bulb of the degassing tower by means of a 2-mm. bore vacuum stopcock. Next, the bulb containing the oil was cooled in liquid nitrogen until the nitrogen ceased boiling. The system was then reopened to the pump. When the vacuum had returned to the original range (10^{-4} mm.), the U tube was bathed in liquid nitrogen, the oil allowed to warm to room temperature and degassing begun with 80° C. water circulating through the jacket. Four hours of recirculating the oil in the degasser was found sufficient to completely deodorize the oil. The sample trapped in the U tube represented a composite of inorganic (H_2O, CO_2, CO) and organic compounds. These contents were displaced to a bulb at -196°

C. by heating the U tube with a hair dryer. Removal of the inorganic compounds using the methods of Bazinet and Merritt (45) and Merritt et al. (39) were successful insofar as removing carbon dioxide and carbon monoxide were concerned. However, good separation of water from the flavor components was never realized. Apparently, the flavor components are highly water soluble and have vapor pressures very near that of water itself. Thus, gaseous samples introduced onto the gas chromatograph were so diluted with water vapor that quantities introduced onto the column were too small for good preparative separations.

Steam Distillation Techniques. Approximately 4 pounds of freshly prepared peanut butter were slurried with distilled water and added to the 12 liter flask along with additional water to provide a total volume of about 8 liters. The mixture was brought to a boil with rapid stirring and distilled concurrently with the hexane-ether mixture. Continuous return of water to the distilling flask and hexane-ether to the solvent flask was provided for in the design of the continuous extractor illustrated in Figure 3.

When the organic colvents were removed, a viscous, oily, highly odoriferous residue remained. The yield of this residue was about 0.03 ml. per pound of ground peanuts distilled.

Fractionation and Chromatography of Steam Distillates. The viscous residue was transferred to a tube suitable for "bulb to bulb" distillation and equilibrated first at -80° C. with a -196° C. bulb for 2 hours. Secondly, it was equilibrated at room temperature with another -196° C. for 6 hours. The first distillation removed traces of organic solvents remaining in the steam distillate and at least one other compound. Both fractions were analyzed by mass spectrometry at both high (70 volts) and

low (20 volts) voltage. Increases in magnitude of parent mass peaks are normally relatively greater than those for other peaks when lower voltages are used. In this way it was possible to establish the number of components in each fraction and their parent masses.

The material which distilled at room temperature was then separated into individual components by preparative gas chromatography. This amounted to repetitively making 50 µl. injections and collecting sufficient volume of each eluant for spectrometric studies.

Mass Spectral Analyses and Interpretation. Analyses of the gas chromatographically separated samples were performed as follows: the collection tubes were connected to the gas inlet system of the mass spectrometer through a hypodermic needle, cooled to -196° C. and the entire inlet system including the tube pumped down to about 0.1 microns pressure. Next, the temperature of the collection tube was allowed to rise slowly. This allowed the gases to expand into the inlet system of mass spectrometer. Good spectra were obtained if as much as 50 microns pressure could be obtained in the inlet.

Spectra of composite samples were obtained using the procedures outlined by Bazinet and Merritt (45). Identifications from the spectra were made using the procedures of Bazinet and Merritt (45) and Rock (63).

Preparation of Peanuts for Oil Extraction and Steam Distillation. Peanuts were roasted 4 pounds at a time in an electrically heated, rotating wire basket. The peanuts were cooled, split in a laboratory splitter, the testa and the germ removed and the roasted cotyledons finely ground into peanut butter.

RESULTS AND DISCUSSION

Study of Samples from Degassing of Peanut Oil. Gas chromatograms of the composite sample obtained by degassing peanut oil revealed ten peaks. One, peak 10, was later shown to contain two components making a total of eleven components (Figure 6). Quantities of material introduced onto the column were too small to obtain reliable mass spectra of collected fractions. However, mass spectra of composite samples were obtained by fractionally distilling directly onto the mass spectrometer. The peaks found in spectra of the composites were always consistent with those found in individual fractions. Sufficient sample was obtained in these cases to obtain mass:charge ratios (m/e) of considerable magnitude. However, these were still spectra of multicomponent systems and could not be used for positive identifications. Tentative identifications (Table 1) were made from parent mass and high mass peaks.

Compounds responsible for peaks 5 through 10 (Figure 6) contained distinct odors reminiscent of roasted peanuts while compounds representing the first four peaks were odorless as they emerged from the exit port.

Numerous problems were encountered with the technique of trapping components during chromatography of composite samples obtained by degassing Eluted components were only partially recovered even when the collection tubes were bathed in liquid nitrogen. Shrinkage of silicone-gum rubber is appreciable at -196° C. and the use of septums made from this material had to be abandoned. Rubber septums did not shrink appreciably but did often fail to close after being punctured by the fraction collector needles.

Other troubles were also encountered. Peanuts stored in contact

with chemical vapors will absorb sufficient quantities to completely mask flavor components in the mass spectra and gas chromatograms even when stored in closed, plastic containers. Also, inability to separate water from the remaining volatiles of the composite samples contributed measurably to the failure to obtain positive identifications using this procedure.

Study of Steam Distillates. Steam distillation of freshly prepared peanut butter completely removed the typical aroma and taste of roasted peanuts yielding approximately 0.03 ml. of a viscous, yellow, typically odoriferous, oily residue per pound of peanut butter distilled. The thick aqueous suspension of peanut butter remaining tasted much the same as flour-water paste.

Attempts to separate the components of this residue by preparative chromatography were not completely successful. About 50% of the material injected onto the column was recovered as ethanol and water; yet, infra-red and NMR spectra showed the complete lack of any hydroxyl functional groups in the residue before injection. Besides ethanol and water, two other compounds were present in considerable quantity. These were identified by mass spectrometry as 2,5-dimethylpyrazine and benzaldehyde. The infra-red and NMR spectra of the residue before injection showed no indications of aromatic nuclei or aldehyde groups. When the distillate collected from the room temperature to -196° C. distillation was chromatographed, the tracing was nearly identical to that obtained from the total steam distillate residue except that ethanol and water were no longer present. Benzaldehyde and 2,5-dimethylpyrazine peaks were still present. Infra-red and NMR spectra of these distillates were those expected for aldehyde functional groups and heterocyclic nitrogen

compounds. Evidently, the water and ethanol observed in the early chromatograms of the total steam distillate were formed by reactions occuring on the column and originated from the material which did not distill during the room temperature to -196° C. distillation. This residue remaining in the room temperature bulb was not devoid of aroma, but was nearly so when compared to the material in the -196° C. bulb.

Mass spectra of the material distilling during the -80° C. to -196° C. equilibration showed that this operation removed the last traces of hexane and ether from the total steam distillate. However, some benzene was also identified with confidence. Although the presence of benzene as a natural constituent of peanut aroma seems unlikely, it had been identified on two different occasions during analysis of samples obtained by degassing of oil. At that time, its presence was attributed to impurities absorbed by the peanuts.

Of the total steam distillate, only about 15% distilled to the -196° C. bulb at room temperature. This meant that the yield of flavor concentrate was only about .0045 ml. per pound of peanut butter originally distilled. Therefore, in order to obtain sufficient amounts of each of the twenty or so components present in this concentrate for infra-red as well as NMR measurements, several hundred pounds of peanuts must be processed. If instrumental analyses do not supply rigorous structure proofs, several thousand pounds of peanuts may have to be processed to obtain enough material for structure proof by syntheses and degradations.

SUMMARY

Apparatus and techniques have been developed or modified and have been used to isolate the substances responsible for the typical aroma

and taste from roasted peanuts. The use of these techniques to isolate and identify the individual flavor components has given only limited success chiefly because of the lack of a sufficient quantity of material. In spite of the difficulties encountered, several tentative identifications have been made and the presence of benzaldehyde and 2,5-dimethylpyrazine have been confirmed with considerable confidence.

ACKNOWLEDGEMENTS

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TABLE I

TENTATIVE IDENTITIES OF SOME VOLATILE COMPONENTS OF ROASTED PEANUTS AS DETERMINED BY MASS SPECTROMETRY

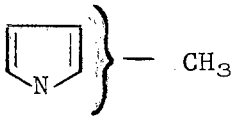
Acetylene	$HC{\equiv}CH$
Hydrogen cyanide	HCN
Propiolonitrile	$CH{\equiv}CH\text{-}CN$
Tetrahydrofuran	
Methyl pyrrole	
Methane thiol	$CH_3\text{-}SH$
Butanal	$CH_3\text{-}CH_2\text{-}CH_2\text{-}CHO$
Allyl ethyl ether	$CH_2{=}CH\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH_3$
2-Methyl propanol	$CH_3\text{-}\underset{\displaystyle CH_3}{\underset{\vert}{CH}}\text{-}CH_2OH$

Figure 1. Schematic drawing of the manifold system and bulbs used for degassing and "bulb to bulb" distillations

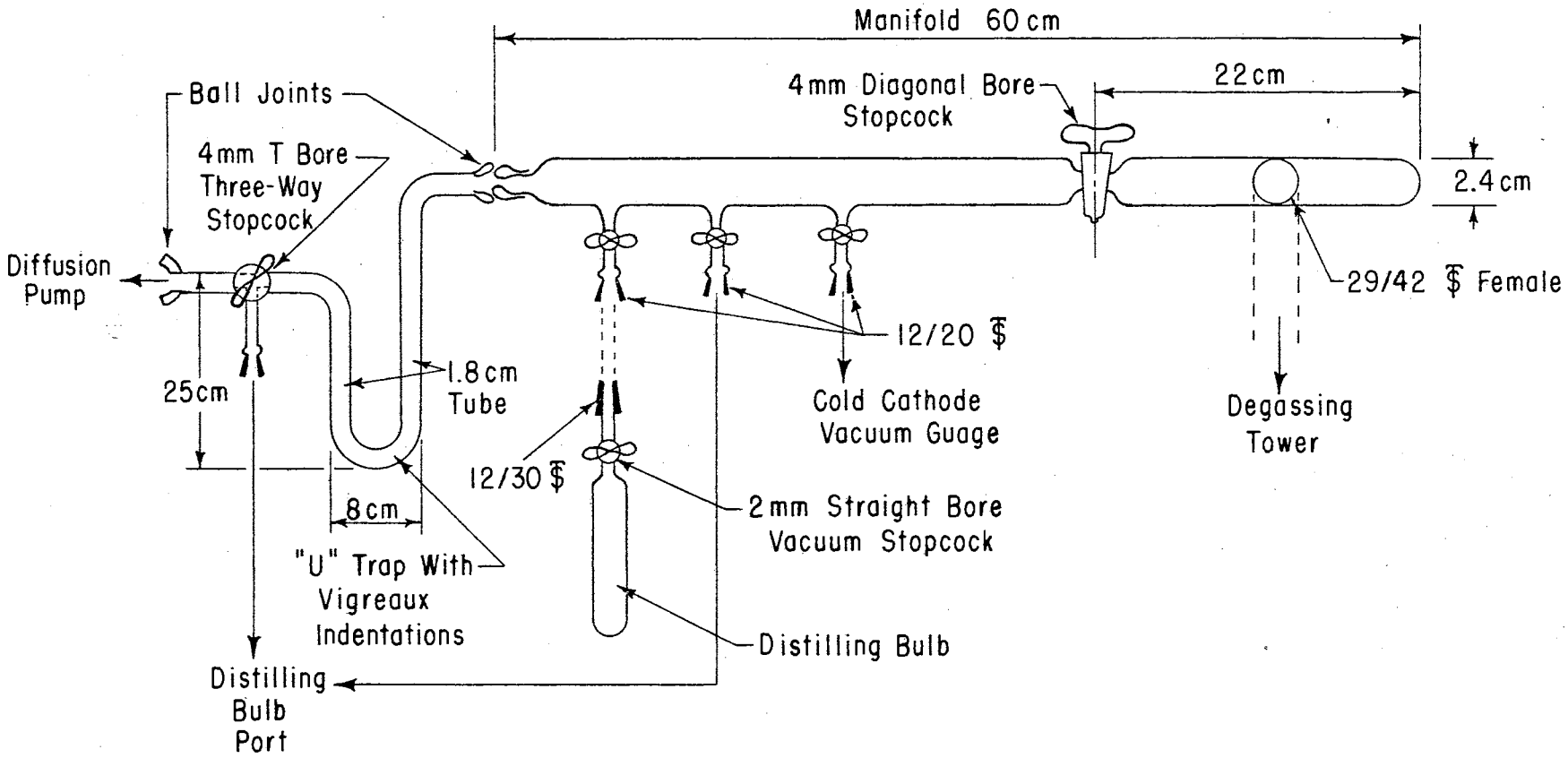

Manifold 60 cm
22 cm
4 mm Diagonal Bore Stopcock
Ball Joints
4 mm T Bore Three-Way Stopcock
Diffusion Pump
2.4 cm
29/42 Female
12/20
Cold Cathode Vacuum Guage
Degassing Tower
25 cm
1.8 cm Tube
12/30
2 mm Straight Bore Vacuum Stopcock
8 cm
"U" Trap With Vigreaux Indentations
Distilling Bulb
Distilling Bulb Port

Figure 2. Schemtaic drawing of the tower used for degassing of peanut oils

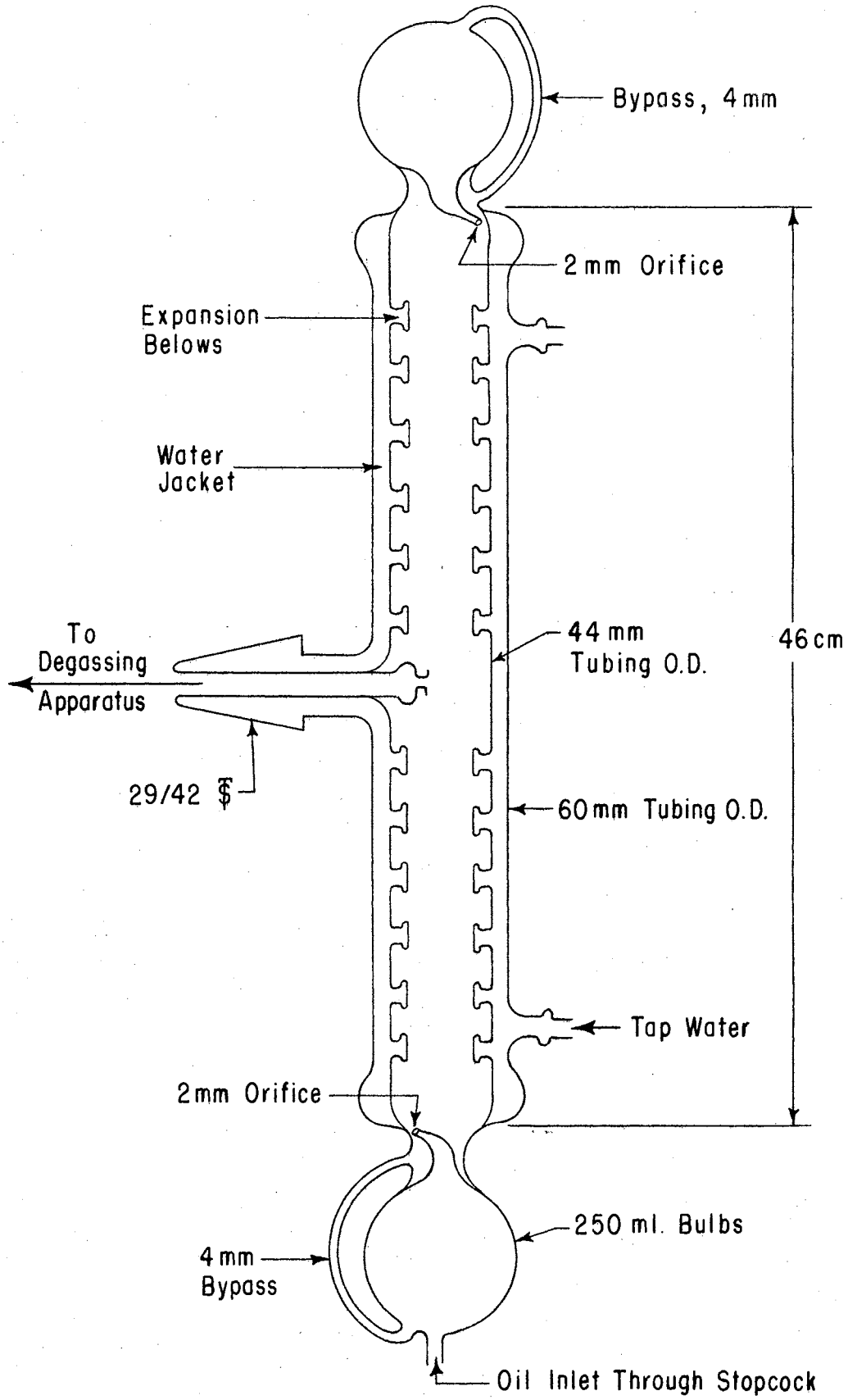

Bypass, 4 mm
2 mm Orifice
Expansion Belows
Water Jacket
To Degassing Apparatus
29/42 $
44 mm Tubing O.D.
46 cm
60 mm Tubing O.D.
Tap Water
2 mm Orifice
250 ml. Bulbs
4 mm Bypass
Oil Inlet Through Stopcock

Figure 3. Schematic drawing of the liquid-liquid continuous extractor used in conjunction with steam distillation

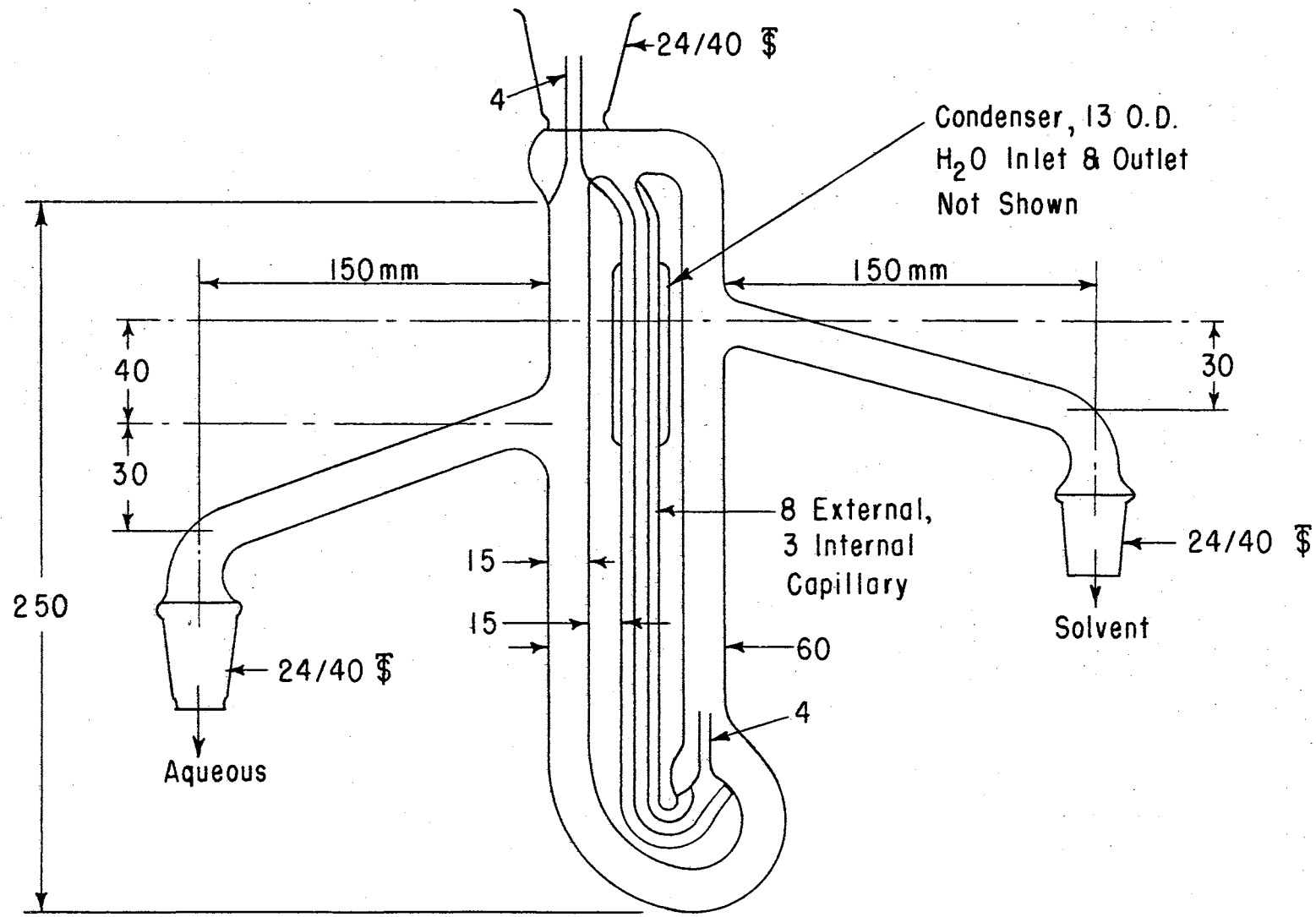

24/40 $
4
Condenser, 13 O.D.
H_2O Inlet & Outlet
Not Shown
150 mm
150 mm
40
30
30
250
8 External,
3 Internal
Capillary
15
15
60
4
24/40 $
24/40 $
Solvent
Aqueous

Figure 4. Schemtaic drawing of collection tubes for trapping components of steam distillates separated by preparative gas chromatography

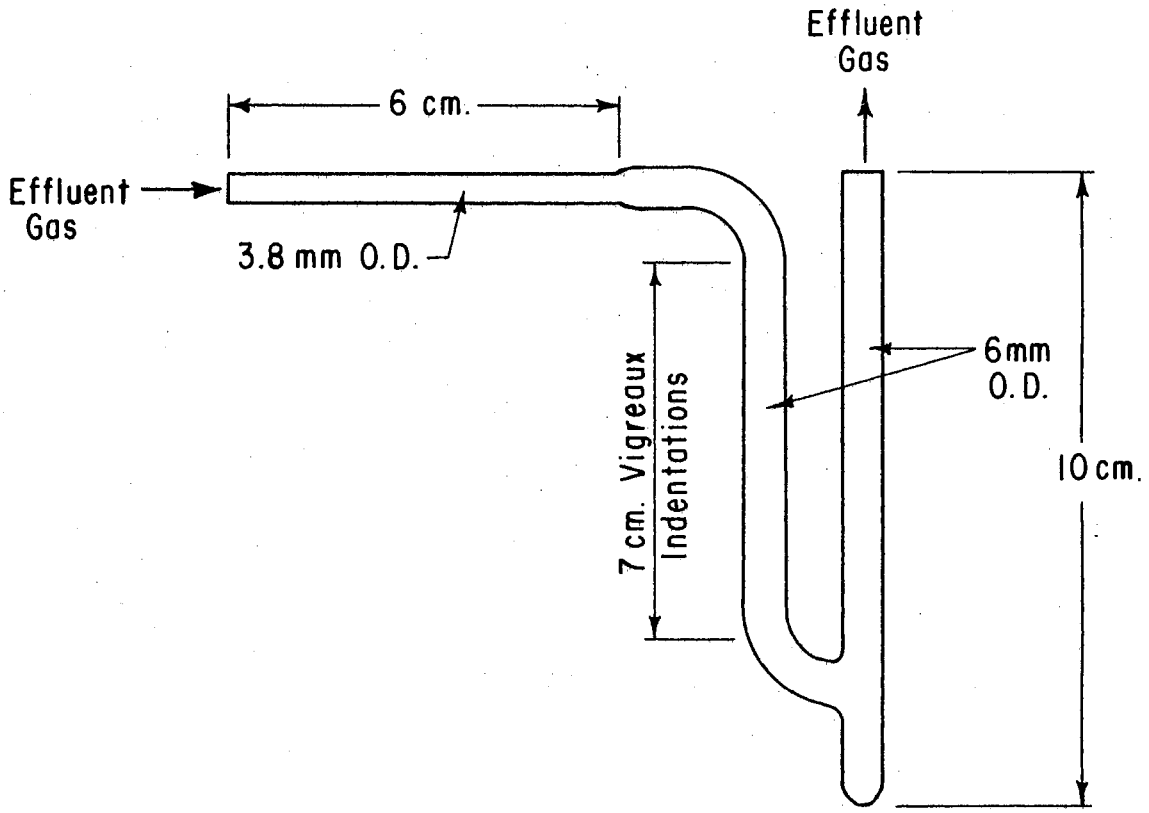

Effluent
Gas
6 cm.
Effluent
Gas
3.8 mm O.D.
7 cm. Vigreaux
Indentations
6mm
O.D.
10 cm.

Figure 5. Schematic drawing of the inlet system used to introduce gaseous samples onto gas chromatographic columns

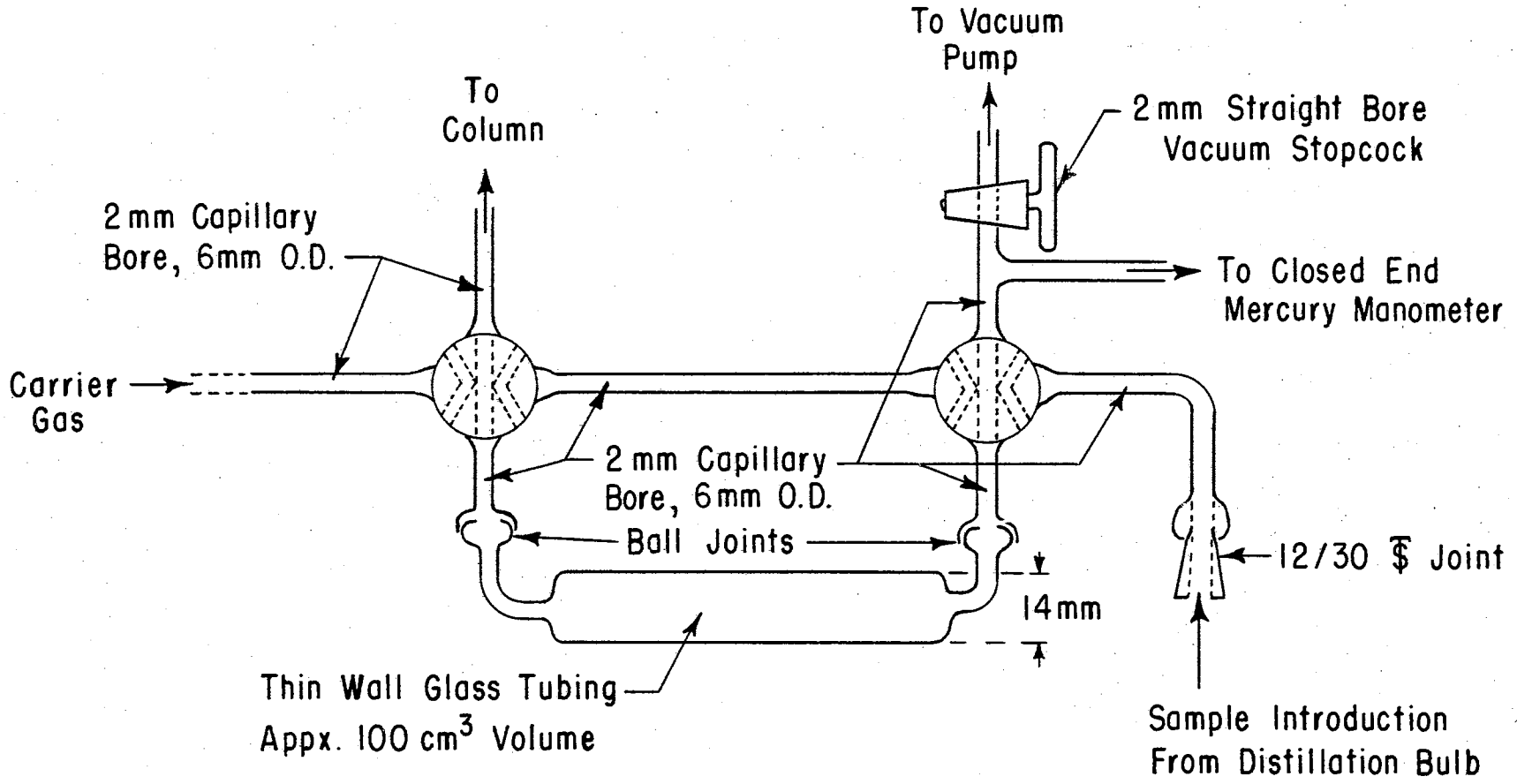
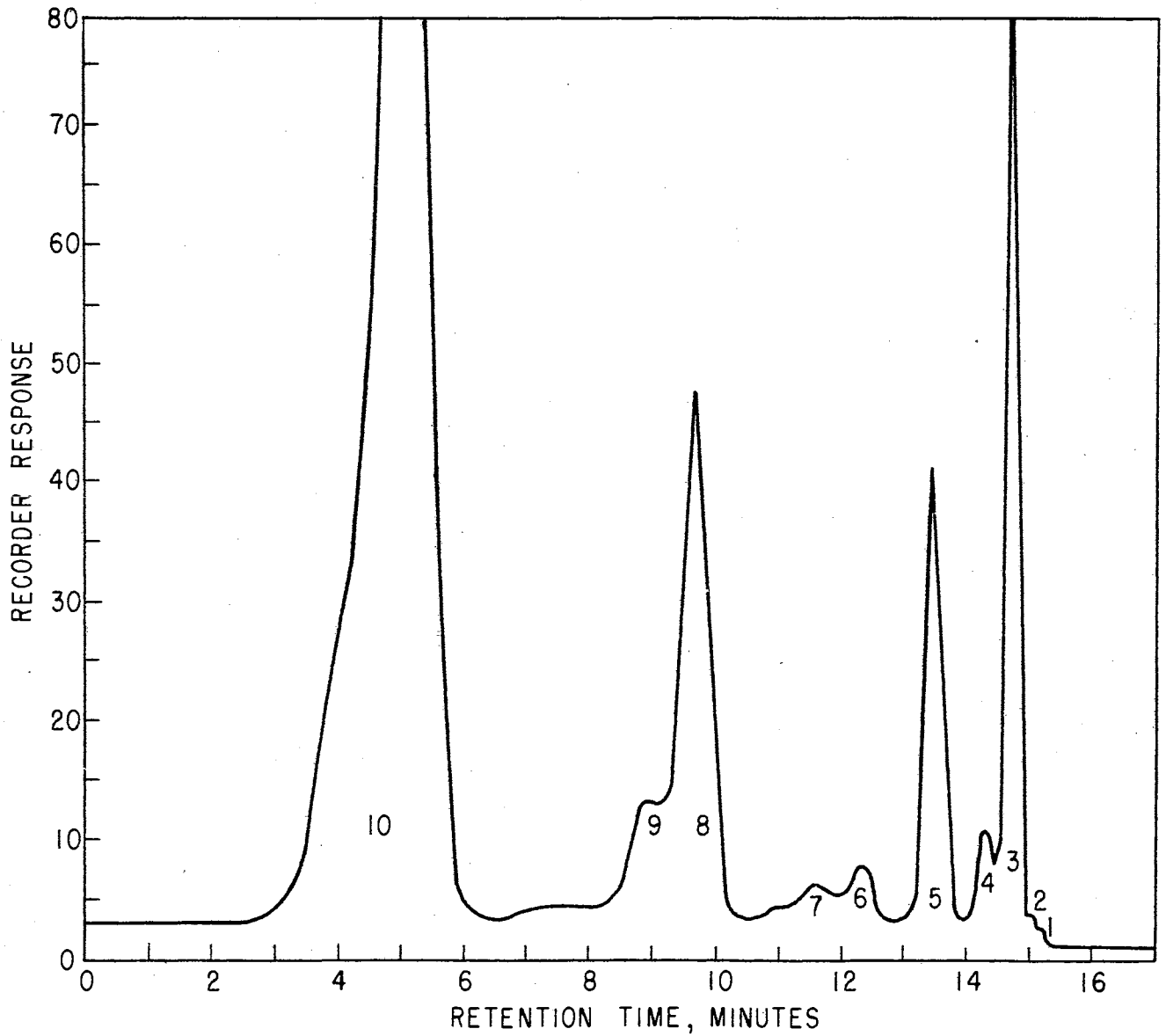

To Vacuum Pump
To Column
2 mm Straight Bore Vacuum Stopcock
2 mm Capillary Bore, 6mm O.D.
To Closed End Mercury Manometer
Carrier Gas
2 mm Capillary Bore, 6mm O.D.
Ball Joints
12/30 Joint
14mm
Thin Wall Glass Tubing
Appx. 100 cm3 Volume
Sample Introduction From Distillation Bulb

Figure 6. Gas chromatogram of composite sample collected by degassing oil pressed from roasted peanuts


80
70
60
50
40
30
20
10
0
RECORDER RESPONSE
0
2
4
6
8
10
12
14
16
RETENTION TIME, MINUTES
10
9
8
7
6
5
4
3
2
1

CHAPTER VII

SUMMARY

Two simple, convenient and quantitative procedures for the preparation of methyl esters of fatty acids from fats and oils have been developed. Gas chromatographic analyses are made by injecting aliquots taken directly from the reaction mixture thus eliminating evaporation and extraction steps used in other procedures. The use of 2,2-dimethoxypropane to drive transesterification to completion eliminates the need for elevated temperatures. DMP reacts with glycerol to form isopropylidene glycerol which chromatograms readily and serves as a convenient marker for determining retention times.

One procedure is especially adaptable to routine analyses of large numbers of samples where only relative values are desired. The other procedure is less convenient but affords additional information. Both glycerol and fatty acid contents are determined. Knowledge of the amount of both components allows a comparison of the weight of the fat determined by analysis with the weight of sample used for analysis. Also, comparison of the μmoles of glycerol with the μmoles total esters found provides an additional check on the purity of the sample analyzed and the precision of the analysis. Glycerol and fatty acid contents may be expressed on an absolute basis (μgm. or μmoles fatty acid per 100 mg. of fat) rather than a relative basis (μmole percent of total fatty acids).

Experimental evidence to support quantitative application of both

of these methods are presented.

Very little is known concerning the structures and location of precursors of roasted peanut flavor within peanut cotyledons. Work reported here represents initial results of attempts to isolate and identify these compounds. The techniques used for these studies have revealed useful information concerning the locale and molecular origin of flavor precursors within peanut cotyledons. Several conclusions drawn concerning the molecular origin of flavor during roasting are: (1) Flavor does not appear to arise from the large globulin proteins (Arachin and Conarchin) and (2) it does not appear to arise from carbohydrate material per se. (3) Flavor does arise from one or a combination of ninhydrin positive and basic compounds.

Apparently, flavor originates from rather specific types of micromolecules rather than the general macromolecular cellular components, such as the large globulin proteins and the starches.

Additional work designed to reveal the chemical identities of the components of roasted peanut flavor have met with only limited success. Nevertheless, peanut butter has been deodorized and typical flavor removed by continuous steam distillation and oil pressed from roasted peanuts has been deodorized by using degassing techniques. Further separation of the steam distillate by low temperature, high vacuum distillation and study of the fractions obtained by nuclear magnetic resonance and infra-red spectroscopy have shown that the aroma components make up only a small portion of the total steam distillate. Calculations based on the yields of these distillates show that several thousand pounds of roasted peanuts will have to be processed to obtain enough aroma components for studies by means other than mass spectra.

Several tentative identifications were made from mass spectral analysis of samples obtained by degassing the oil. Two major components of the steam distillate identified with reasonable confidence using mass spectra were 2,5-dimethylpyrazine and benzaldehyde.

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APPENDIX

POLYMER FORMATION DURING TRANSESTERIFICATION

The first visible phenomenon noted in the transesterification reaction mixtures designed to convert glyceride fatty acids to methyl esters for subsequent quantitation by gas chromatography was the appearance of a yellow color which would develop progressively into orange, purple and even black colors if given sufficient time. This was obviously an unwanted reaction since none of the desired compounds arising by way of transesterification would yield colored products. In fact, it was soon found that this side reaction became predominant if not controlled. The resulting colored compounds were present in sufficient quantities to completely or partially interfere with resolution of methyl esters in subsequent gas chromatograms.

Figure 1 summarizes studies on the effect of 2,2-dimethoxypropane (DMP) and HCl concentration on colored compound formation at 22° C. Clearly, as the DMP concentration increased, the rate of colored compound formation measured at 422 mμ also increased markedly as manifested by the increased slope of the rate curves 1 through 5. However, the HCl concentration became the rate limiting factor as the molar ratio of HCl to DMP became small. This was shown by the slower rate indicated by curve 6 when compared to curve 5. Thus, even though the DMP concentration of the solution measured by curve 6 was greater than that measured by curve 5; the trend was reversed and the rate of colored compound formation decreased slightly.

Increased colored compound formation during transesterification reactions with increased temperature was not explicitly measured, but was implicitly observed from the profound increases in polymer peaks when reaction mixtures which had been submitted to temperature increments were chromatographed. Chromatograms of reaction mixtures allowed to stand overnight at temperatures as high as 30° C. contained colored compound peaks as much as 60 times as large as the methyl ester peaks.

The wavelength of maximum absorbance (λ max. 422 mμ) for colored compound formed from acidified DMP was the same as that formed from acidified acetone. Consequently, it was theorized that the colored compound was a phorone-like, polymeric, condensation product between the enol and keto forms of acetone. In solutions of DMP, the acetone would arise from the acid catalyzed decomposition of DMP in the presence of small amounts of water as outlined by Kreevoy and Taft (64). Explanation for the more rapid formation of colored compound in the presence of DMP than in acetone is found if one further theorizes that DMP has a dual purpose in not only supplying acetone by way of decomposition but also in aiding in the dehydration of condensation products leading to phorone or polyphorone. This thought is consistent with the fact that polymer formation involves the loss of hydrogen and oxygen as shown by elemental analysis. An average empirical formula of $C_{16}H_{18}O$ was found. Also, the infra-red spectrum (Figure 2) of the polymer showed the presence of the carbonyl function along with absorption in the region expected for conjugated double bonds. Two very strong bands absorbing equally at 1362 cm.$^{-1}$ and 1378 cm.$^{-1}$ are indicative of the importance of methyl groups in the structure. Under acid conditions, DMP reacts quantitatively with water to produce acetone and methanol (65). If double bonds were formed

by the elimination of the elements of water, the creation of more acetone from DMP would occur as water was continually eliminated. Accordingly, polymer formation would be self perpetuating and only catalytic amounts of water would be needed in the reaction mixtures to initiate and perpetuate the reaction. This thought is consistent with the data in Figure 1 showing that polymer formation does not cease but continues as though the supply of water were continuous. Observations of color formation for periods of several days on the recording spectrophotometer showed that the reaction rate was fairly constant with no tendency to level off.

Thus, data from the rate studies, infra-red spectra, and elemental analysis are consistent with the theory that the polymer is formed by condensation of acetone under acidic conditions followed by water eliminations; the evidence remains circumstantial.

Figure 1. Polymer formation measured at 422 mμ as a function of HCl and DMP concentrations

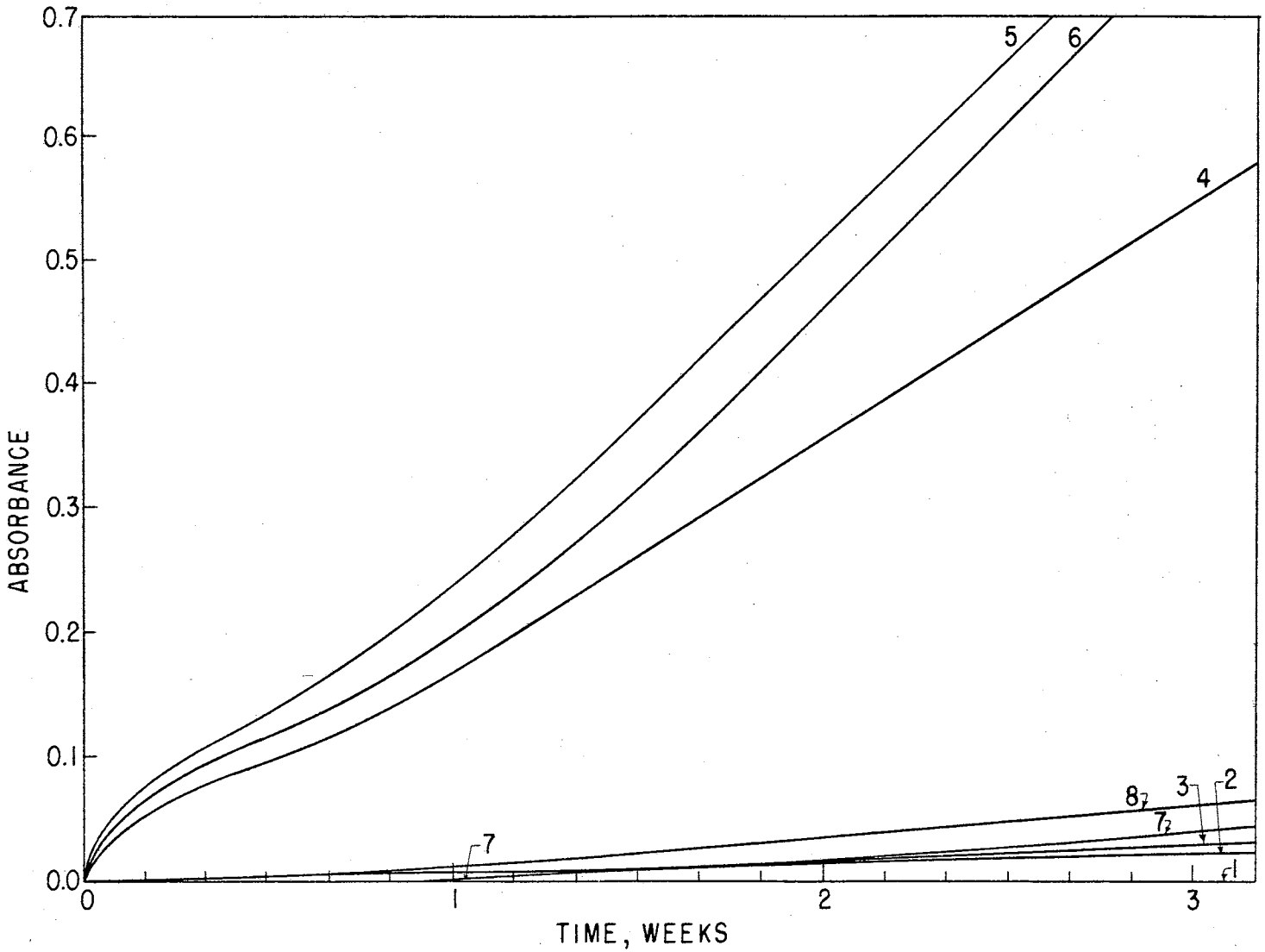
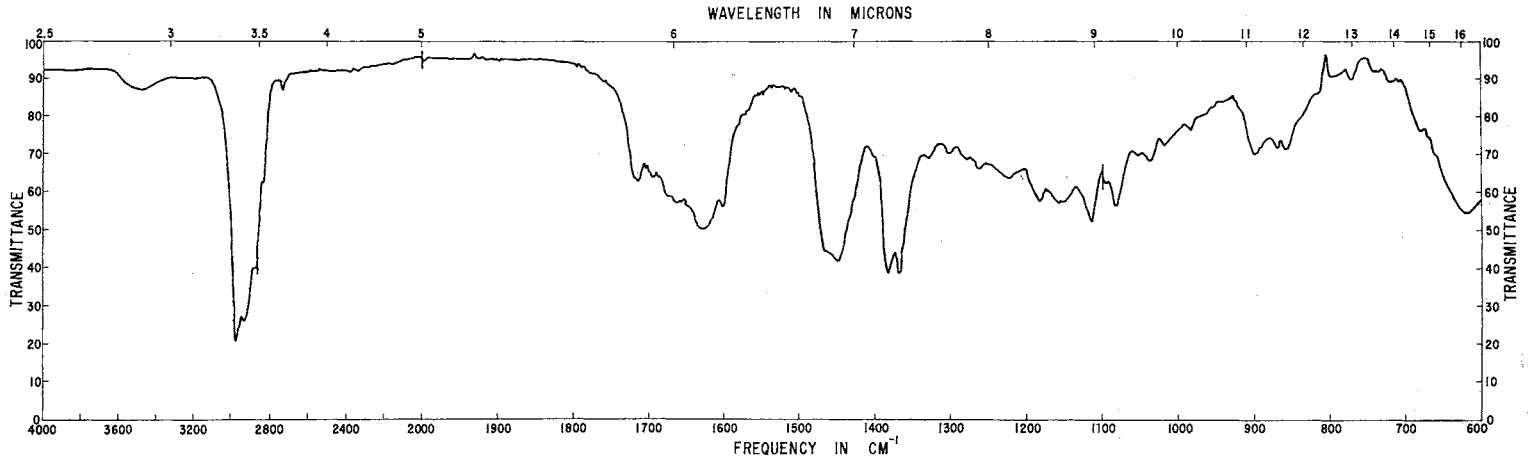

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
0
1
2
3
TIME, WEEKS
5
6
4
8
3
2
7
1

Figure 2. Infra-red spectrum (film) of polymer formed by acidifying DMP with HCl gas


WAVELENGTH IN MICRONS
2.5
3
3.5
4
5
6
7
8
9
10
11
12
13
14
15
16
TRANSMITTANCE
100
90
80
70
60
50
40
30
20
10
0
4000
3600
3200
2800
2400
2000
1900
1800
1700
1600
1500
1400
1300
1200
1100
1000
900
800
700
600
FREQUENCY IN CM^{-1}

VITA

Michael Elmer Mason

Candidate for the Degree of

Doctor of Philosophy

Thesis: PROCEDURES IN STUDYING AND FACTORS INFLUENCING THE QUALITY AND FLAVOR OF ROASTED PEANUTS

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